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(54) Title: USE OF POLYAMINES AS IONIC-CHANNEL REGULATING AGENTS		
(57) Abstract <p>Polyamine compounds are used as agents regulating ionic conductances in cellular membranes. These polyamines are used in blocking, modulating or activating calcium and other cationic channels in neuronal cell membranes and in blocking, modulating or activating calcium channels of a specific type in any cell membrane when such channels are present.</p>		

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10 USE OF POLYAMINES AS IONIC-CHANNEL REGULATING AGENTS

This application is a continuation in part of (a) of U.S. Patent Application Serial No. 219,105 filed July 14, 1988 (now allowed) in turn a continuation-in-part of U.S. Patent Application Serial No. 154,845 filed February 10, 1988, (now abandoned); (b) PCT Application PCT/US89/00558 (published under WO89/07608) filed on February 10, 1989 (now U.S. Application Serial No. 435,488 filed October 10, 1989), and (c) U.S. Application Serial No. 375,776, filed July 3, 1989. The United States Government has rights in this invention by virtue of Grant No. NS-13742 from the National Institute of Neurological and Communicative Disorders and Stroke and NIH EY-08002 from the National Eye Institute, U.S. Public Service, National Institutes of Health.

25 Field of the Invention

This invention relates generally to the use of polyamine compounds as agents regulating ionic conductances in cellular membranes. More specifically, certain aspects of this invention relate to the use of certain polyamines in blocking, modulating, or activating calcium (as well as other cationic) channels in neuronal cell membranes and in blocking, modulating or activating calcium channels of a specific type in any cell membrane where such channels are present.

30 Background of the Invention

35 Passive transport of charged particles across cell membranes, in response to an incremental change in an electrical field across the thickness of the cell membrane, is mediated (and, in substantial part, regulated) by membrane

channel proteins (including those referred to as "voltage-sensitive" or "voltage-dependent" calcium channels).

In this discussion the terms "channel" and "channel protein" are used interchangeably without implying that a channel must necessarily consist of a single protein, although the channels that have been isolated are believed to be single proteins.

Most channel proteins mediate the transport of one ionic species with substantially higher specificity than transport of other ionic species. It is common to name various channels after the ion for which they are specific: thus, there are sodium channels, potassium channels, calcium channels, etc.

In turn, ionic channels specific for the transport of one cation, may be further divided into various subcategories or channel types, based on the way they interact in response to electrical and/or chemical (pharmacological) stimuli. Calcium channels in particular, which have been identified in a number of different cell types, including neurons, appear to have differences (as well as similarities) in morphology, properties and/or function. Some of these differences are ascribable to the morphology and function of the cells in which such channels occur: for example neuronal calcium channels are by far the most complex in all three aspects. Miller, R.J., infra. Other differences have not been directly related to cell types; in fact, different types of calcium channels are normally present in the same cell. Neuronal cells, for example, display four operationally distinct types of calcium conductance and thus there are four "types" of neuronal calcium channels. (Some of the properties of each type of neuronal calcium channel, however, appear to be shared by calcium channels in other tissues.)

Historically, neuronal calcium conductances were first divided into two categories based on the driving force that activates them: the high-threshold calcium conductance (or HTCC) and the low-threshold calcium conductance (LTCC). In central neurons, HTCC is more prominent in the dendrites and LTCC is more prominent in the soma or cell body. Later,

neuronal calcium channels were grouped into three categories: the T-channels, which are believed to be responsible for LTCC; the N-channels, the conductance properties of which showed imperfect correspondence to HTCC; and the L-channels which are not commonly represented in central neurons but of which the conductance properties also showed correspondence to HTCC.

These three categories can be further distinguished by differences in pharmacological properties. The L-channels are dihydropyridine-sensitive, i.e. they are effectively blocked by dihydropyridines, such as nifedipine and nitrendipene, whereas the T- and N-channels are dihydropyridine-resistant. To a large extent, the T-channels resist blockage by cadmium ions; more important, the T-channels are specifically blocked by alcohols (especially octanol) at 10^{-4} M or lower concentrations. Finally, both the N- and L-channels are said to be blocked by omega-conotoxin, a toxin isolated from the venom of the marine snail Conus geographicus to which the T-channels are resistant. Miller, R.J. Science, 1987, 235: 46-52.

However, the calcium channels responsible for the HTCC (both the calcium-dependent plateau potential component and the dendritic spike component of the HTCC) in Purkinje cells are activated at -50 mV, and are dihydropyridine-insensitive and also omega-conotoxin-resistant. These channels are specifically blocked by a low-molecular weight blocking agent isolated from the venom of funnel-web spiders. Llinas et al, PNAS, 1989, 86:1689-1693. (Specificity is confirmed by the fact that this blocking agent does not block any of the conductances attributed to the T-channel, the sodium channel, the dihydropyridine-sensitive channel, the conotoxin-sensitive channel, or the potassium channel except insofar as the conductance of the latter is regulated by calcium.) Furthermore, the dihydropyridine- and conotoxin-resistant calcium channels appear to be absent from inferior olivary and thalamic neurons. These calcium channels have been called P-channels because they were first described in Purkinje cells. Llinas, R.R., et al., Ann. N.Y. Acad. Sci., 1989, 560:103-111. P-type

channels have also been shown to exist in squid giant synapse and squid optic lobe.

Sodium and potassium channels are also of various types. See generally Hille, B. ["Ionic Channels of Excitable Membranes" Sinauer Assoc. Inc., Sunderland, Mass. 1984.]

Two main types of sodium channels are found in electrically excitable cells such as central neurons. One type of channel, responsible for the so-called fast sodium conductance, is specifically blocked by tetrodotoxin (TTX), a toxin isolated from puffer fish. A second type of sodium channel, responsible for the slow sodium current is blocked by local anesthetic agents such as lidocaine. A distinctly different sodium channel is located in non-electrically excitable tissue such as epithelium, and is not blocked by TTX or the local anesthetics but blocked by the diuretic amiloride.

In contrast, many types of potassium channels are thought to exist. While a few types of potassium channels are specifically located in neurons, many are ubiquitously disseminated throughout the body. The known blockers of K⁺ channels, tetraethylammonium (TEA), the amino-pyridines and quinine show little specificity towards any particular type of potassium channel.

Agents that block calcium channels with high affinity and specificity as well as agents that activate calcium channels are useful as reagents in electrophysiological research. Availability of such agents is essential for understanding calcium channel properties and function. Such agents are especially useful in the design of prototype drugs and in drug screening.

For example, a blocking agent specific for P-channel permits the investigation of conductances of other channels (which coexist with the P-channel in various in vitro or in vivo experiments) without interference from the P-channel calcium conductance. In addition, such a blocking agent can serve as a prototype drug which could be used, e.g., for regulating calcium transport through cellular membranes. In turn, such a drug would have potential applications in prevent-

ing cell death caused by ischemia or other anoxic cerebro-
pathies or by other factors which disrupt the regulation of
calcium transport, such as aging. Such drugs also would have
potential applicability in the treatment of epilepsy, and
5 memory and learning disorders.

The prior art calcium-channel blocking agents do not
satisfy this need for one or more of the following reasons:

(a) Lack of Affinity for Particular Calcium Channels.
The P-channel in particular is resistant to the natural toxin,
10 omega conotoxin; to dihydropyridine and its derivatives; and to
alcohols.

(b) Lack of Specificity. When developing substances that
block a certain channel, specificity is a primary concern.

(c) Unavailability in Large Amounts at Low Cost. It
15 would be desirable to identify the chemical structure of the P-
channel blocking agent derived from funnel-web spider venom as
well as the structure of active derivatives and analogues
thereof as well as other specific P-channel blockers which
could be synthesized conveniently and at low cost.

20 (d) Lack of Knowledge of Blocking Agent Mode of Action.
The availability of specific P-channel blocking agents would
make it possible to identify the site of blocking activity and
to design improved substitutes or even compounds having the
opposite property: opening calcium channels.

25 Calcium-channel activating agents (i.e. agents that
increase the influx of calcium through these channels) are even
more scarce than calcium-channel blockers. A known L-channel-
specific activator is available commercially under the
tradename Bay-K8644. However, there are no prior art ac-
30 tivators for N-, T- or P-channels. Thus, there is an even more
acute need for specific, high-potency and/or inexpensively
available calcium channel activators than for calcium blocking
agents.

35 Specific calcium-channel activators can also serve as
useful research reagents, but unlike blockers, activators can
be used to test different channel properties such as, for
example, the limits of the capacity of P-channels to allow

calcium influx into a cell. Activators can also be used to study synaptic transmitter release and other aspects of transmission more closely, e.g., by amplifying the driving force, namely the presynaptic inward calcium current.

5 The Ca channel activators of the present invention also have potential uses as prototypic drugs exhibiting anticonvulsant (e.g. anti-epileptic), anxiolytic, tranquilizing, anti-Alzheimer's, and/or memory-improving properties. More generally activators are potentially useful as prototypic
10 drugs in pathologies or behavioral alterations attributable to an insufficient ability of cells (especially neurons) to permit Ca^{++} influx.

 Analogous needs exist for substances that alter the ion-transport properties of other ionic channels, such as
15 sodium or potassium channels. Such substances also find therapeutic uses such as:

 Alteration of the function of Na^+ channels may be of therapeutic utility for the treatment of muscle spasms, torticollis, tremor, learning disorders, and Alzheimer's
20 disease. Polyamines which block slow sodium channels would have additional utility as local anesthetic agents. Agents which act on the epithelial Na channel would be useful adjuncts in the treatment of cystic fibrosis, and asthma and as antihypertensive agents.

25 Drugs which modulate the activity of K^+ channels would be useful as protective agents against the damaging effects of anoxic and ischemic disorders and hypertension, act to protect red blood cells against damage in malaria, and sickle-cell disease.

30 Calcium activators and calcium blockers (as well as other types of calcium modulators), used separately, are expected to yield information about how the event of cell death is organized. For example, the influence of the presence of a calcium activator in the extracellular medium on the onset and
35 rate of progression of the cytosolic calcium "flood" observed could be measured, eliciting information on whether an increased ability of the cell membrane to transport calcium

influences the onset and the rate of release of intracellular calcium and, more important, whether this late-occurring manifestation of cell death can be reversed.

Chideckel, E.W., et al., Br. J. Pharmacol., 1986, 89:27-33 report that transient exposure to "polyamines" causes a relaxation of guinea-pig respiratory tract smooth muscle (trachealis muscle) in response to subsequent exposure to potassium ion extracellularly. This is contrary to the normal contractile response of this muscle exhibited in the absence of polyamines (or other calcium-ion entry blocking drugs such as nifedipine, verapamil, diltiazem or calcium ion-free solutions). The authors hypothesize inter alia that spermidine may have a Ca^{++} antagonist function and that spermidine and certain other polyamines may have a calcium channel blocking activity, "perhaps" similar to that of the known Ca^{2+} -entry blocking drugs referred to above.

Spermidine was the only polyamine tried in the experiments described in this article, although effects of other polyamines related to membrane Ca^{++} fluxes and cellular Ca^{++} handling are cited from other literature as follows:

- * putrescine is said to cause an increase in cytosolic Ca^{2+} concentration in heart and kidney slices attributed to both an increase in extracellular Ca^{2+} influx and a simultaneous release of Ca^{2+} from mitochondria;
- * spermine is said to inhibit spontaneous contraction of the uterus muscle, in a manner than can be counteracted by increasing extracellular Ca^{2+} concentration;
- * spermine and spermidine are said to have a relaxant effect on smooth muscles of the gut, uterus, respiratory tract and vasculature.

The authors do not mention the type of calcium channels that are said to be present in trachealis muscle (or for that matter in the other tissues mentioned.) As to trachealis muscle, dihydropyridine sensitivity indicates that L-type channels might be implicated. As to the other tissues

mentioned, no claim is made in this article that calcium channels were involved (many other factors or processes could influence the concentration of calcium in the cytosol). In fact, some of the results reported in this article are inconsistent with blockage of calcium channels, and (based on the disclosure of this article) cannot be attributed to activation of calcium channels because of the many other factors that may be at work, including actions on potassium channels or directly on smooth muscle (papaverine-like effects).

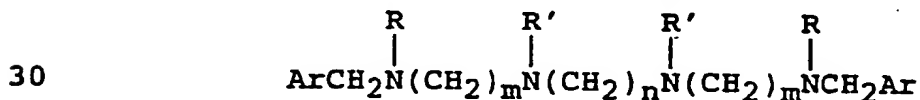
Hirsh, S.R., et al, Psychopharmacology, 1987, 83:101-104 report that two polyamines, spermine and spermidine, administered intraperitoneally, at doses of 5-40 mg/kg, in rats caused a dose-dependent inhibition of spontaneous climbing and wheel running behavior, which the authors tentatively attributed to modulation by these polyamines of limbic dopamine function.

Injected intracerebrally (in the striatum), these compounds failed to induce asymmetric behavior. On the other hand, these compounds did antagonize hyperactivity (when injected into the nucleus accumbens) which was considered consistent with modulation of dopamine function but not dopamine receptor blockage. No effect on ionic (or specifically calcium) conductance is disclosed or suggested.

Palade, P., 1987, J. Biol. Chem. 262:6149-6154 reports that the release of Ca^{2+} (pre-loaded into isolated subfractions of sarcoplasmic reticulum) usually observed upon addition of various release-inducing substances (including caffeine and thymol) could be blocked by ruthenium red, certain organic polyamines (spermine, spermidine and triethylene tetramine) certain antibiotics (neomycin, kanamycin, tobramycin, gentamicin, streptomycin and clindamycin) and certain polypeptides (polylysine, polyarginine, some histones and protamine). The authors observed that these agents have only one feature in common: the presence of several amino groups. Based on the inability of the polyamines to affect calcium pump function, the authors concluded that the effect of ruthenium red, spermine, neomycin and polylysine (which hav

quite diverse structures) was not due to interference with the Ca^{2+} pump but to blockage of the sarcoplasmic reticulum calcium channels. Many of these agents appeared to block calcium release at (estimated) nanomolar concentrations. The authors also remarked that calcium release inhibitory potency appeared to be related to the number of amine groups present in a compound, based on the fact that the antibiotics appeared to be more potent on a molar concentration basis. The article discloses no determination on the specificity of these agents for sarcoplasmic reticulum (SR) calcium channels (compared to other SR ionic transport components and/or receptors) although it states that the small concentrations at which these agents are active would "suggest potential utility as probes of sarcoplasmic reticulum calcium channels". However, the authors admit that ruthenium red is not specific and neither is spermine. Moreover, since the experiments reported in this article measured only blockage of calcium release, they did not positively establish calcium channel involvement. The reported results are limited to Ca^{2+} channels in the sarcoplasmic reticulum which have a large conductance (perhaps an order of magnitude greater than other known calcium channels) and, as stated by the authors, may be further limited to one type of Ca^{2+} channel in the sarcoplasmic reticulum. The authors explicitly state that these compounds appear to be active on SR calcium channels that are insensitive to inositol triphosphate.

In a series of papers, Melchiorre, C., et al., report that certain polymethylene tetramines of the formula



wherein Ar is an aromatic group; R, R' are hydrogen or methyl and m, n are various integer combinations within the range 5-14 have M-2 muscarinic receptor blocking activity in guinea-pig heart atria and intestinal ileum. Several of these compounds are said to selectively block the atrial muscarinic receptor with considerably higher affinity than the ileal receptor, and thus could possibly serve to distinguish between the two

receptors. Nothing is disclosed about calcium channels, but a general synthetic scheme for the non-aromatic moieties of the disclosed polyamines is provided: J. Med. Chem., 1989, 32:79-84; J. Med. Chem., 1985, 28:1643-1647; and Can. J. Physiol. Pharmacol., 1980, 58:1477-1483.

Objects of the Invention

The present invention has the following objects:

- to devise novel agents and methods for regulating (e.g. blocking, modulating or activating) ionic channels (including calcium, sodium, and potassium channels) particularly calcium channels and more particularly calcium channels of the P-type;

- to devise novel agents and methods for regulating transmitter release or synaptic transmission;

- to devise additional agents and methods for blocking one or more of the foregoing channels that overcome one or more of the disadvantages associated with known blocking agents described above;

- to use each of such regulating agents and methods to design prototypical drugs that block, modulate, or activate calcium, sodium, and/or potassium channels (with particular emphasis on drugs that block or activate channels of central neurons); and to increase the understanding of ionic channel structure, properties and function;

- to design more active, less costly and/or otherwise improved substitutes for such agents and/or drugs;

- to design agents that have calcium, sodium, and/or potassium channel modulating functions other than activation or blockage. For example, agents which act on the G-protein in cell membranes alter the activity of ionic channels indirectly.

Other objects of this invention will be apparent to those skilled in the art in light of the present specification, claims and drawings.

Brief Description of the Drawings

Figure 1A is a tracing of spontaneous firing activity observed in the guinea-pig Purkinje cell pursuant to applica-

tion of a direct square current pulse (0.3 nA, 62 msec) in the absence of any blocking agents (control).

Figure 1B is a tracing of the response of the Purkinje cell to a 0.45 nA direct square current pulse after
5 addition of spermidine: the calcium conductance is blocked.

Figure 1C upper trace is a tracing of the response of a Purkinje cell to a 0.45 nA direct square current pulse after (i) the P-channel has been blocked with spermidine, (ii) the potassium conductance has been blocked with TEA, (iii) the
10 sodium conductance has been blocked with TTX. The remaining spike is due to a calcium channel of the dihydropyridine-sensitive type. This is demonstrated in Fig. 1C lower trace where the L-channel also is blocked by dihydropyridine or Fig. 1D (lower trace) where the L-channel is blocked by streptomycin. (The upper trace in 1D is of the same type as in 1C.)
15

Figure 2 is a graph of the presynaptic calcium current in the squid synapse observed after addition of various amounts of A. aperta venom in a voltage-clamp experiment demonstrating dose-dependence of this effect.

Figure 3 is a graph showing the fraction of the excitatory postsynaptic potential (EPSP) remaining (with the control taken as 1) after addition of various amounts of partially purified P-channel blocking factor from A. aperta venom in squid synapse. The results demonstrate the dose-
20 dependence of the effect by the factor on the EPSP.
25

Figure 4 shows the postsynaptic action potential in squid synapse pursuant to direct stimulation of the presynaptic terminal. Tracing A was recorded prior to the addition of compound E in the bath (0.15 ml of the preparation of Example
30 2); tracing B was recorded 4 minutes after compound E addition; and tracing C was recorded 6 minutes after addition of compound E.

Figure 5 upper trace is the postsynaptic potential (EPSP) response in squid synapse in a voltage clamp experiment
35 before (A) and after (B) application of spermidine in the bath (which already contained TTX and 3-aminopyridine). The middle trace is the presynaptic calcium current before (A) and after

(B) application of spermidine in the bath. The lower trace is the applied voltage step (28 mV, 5.5 msec).

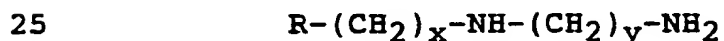
Figure 6 is the trace of the postsynaptic potential again in squid synapse after application of a 38.2 mv/10 msec voltage step in a voltage clamp experiment in the presence of TTX and 3-aminopyridine. Recordings were made 2, 6, 10 and 13 minutes after the addition of spermidine.

Figure 7A and B are traces of the presynaptic and postsynaptic potential in squid synapse in the absence (upper trace) or presence (lower trace) of calcium blocker.

Figure 8 is a superimposition of traces of the inward calcium channel (traces I-1 through I-4) of the presynaptic cell to which a 35 mv/ 1 msec voltage is applied (tracing V) and the corresponding EPSP's (tracings P-1 through P-4) in the presence of TTX and TEA and a calcium channel activating agent, a compound produced by the reaction of lysine ethyl ester and spermidine.

Summary of the Invention

One aspect of this invention is directed to a method for regulating cation transport across cellular membranes possessing cation channels comprising exposing a cell membrane possessing an ion channel of a specific type, to a nonaromatic polyamine compound specifically effecting the channel and having the formula.



wherein y is an integer from 1 to 15; x is an integer from 0 to 15; R is a nonaromatic organic group containing at least one amino, imino, amido, imido and/or may be appended to the remainder of the formula via the group -CX₂-O-NH- (wherein X is H or one of the X's may be NH₂) with the proviso that the distribution of at least one of the methylene groups or nitrogen atoms about the molecule of said polyamine is asymmetric and that the compound contain at least three nitrogen atoms; said polyamine being present in an amount effective to block, activate, or otherwise modulate conductance attributable to this channel.

In addition, the present invention is directed to the compounds of the foregoing formula themselves.

Other aspects of this invention are directed to a methods for blocking (or activating as the case may be) calcium
5 channels and to methods for blocking (or enhancing) synaptic transmitter release using one or more of the compounds referred to above, and/or a polyamine P-channel blocking agent isolated from the venom of the funnel-web spiders.

Another aspect of this invention involves methods for
10 regulating ionic channels, either voltage or ligand-gated such as the glutamate-activated channel, by selecting a compound according to the present invention that is a specific calcium-sodium- or potassium blocker, activator or modulator, and exposing a cell to the presence of this compound to bring about
15 the desired specific regulating result on the target channel.

Detailed Description of the Preferred Embodiments

The disclosure of any and all cited documents including literature and patent applications is incorporated by reference in its entirety.

20 It is understood that in the present specification "ionic channel regulating agents" shall mean compounds and compositions which regulate cation flow by acting directly on a channel or by acting indirectly on it (e.g., by acting on another substance or cellular structure which in turn influen-
25 ces the function of an ionic channel).

Copending commonly assigned U.S. Patent Application Serial No. 219,105 filed on July 14, 1988 discloses that boiling-resistant active factors isolated from the venom of funnel-web spiders by chromatographic techniques, and having an
30 apparent molecular weight of 200-400 daltons on Sephadex G-15 (Pharmacia) size-exclusion chromatography, has the property of blocking calcium channels of the aforementioned P-type with high specificity and affinity. These factors are further characterized by the following properties:

- 35 (a) inactivation in the presence of acid;
(b) resistance to dithiothreitol reduction;

- (c) ability to be coupled to ether-coupled Sepharose 4B (Pharmacia, Piscataway, N.J.) gel.

On structural analysis in accordance with this invention, this calcium channel blocking agent, more specifically a preparation of it isolated from A. aperta spider venom by deproteination (boiling) followed by removal of the precipitate by centrifugation and subjection of the supernatant to FPLC (high-pressure liquid chromatography - Pharmacia) under conditions of 0-1.0 M NaCl, pH 7.5 and a flow-rate of 1 ml/min using a cation-exchange column, e.g., Mono-S (5 x 50 mm) from Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) with the active fraction (resulting from 0.5 ml of venom) eluting at about 0.8 M NaCl, gave the following results:

(a) On UV spectroscopy (using a UV spectrophotometer Milton-Roy Spectronic, Model 1001) the purified A. aperta factor exhibited no optical absorption at 260-280 nm and did not fluoresce, thereby indicating that aromatic rings are absent. The absorption wavelength shifted strongly upon acidification of the sample but remained relatively insensitive to elevated pH. This behavior is consistent with the presence of amine groups.

(b) On FT-IR (Fourier Transform Infrared Spectroscopy) samples were analyzed as Nujol mulls and KBr pellets. The spectra obtained were relatively simple and showed evidence of amine groups as well as methylene groups.

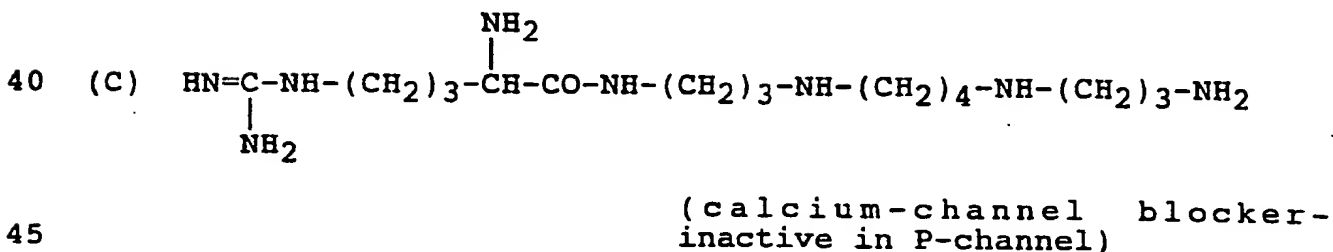
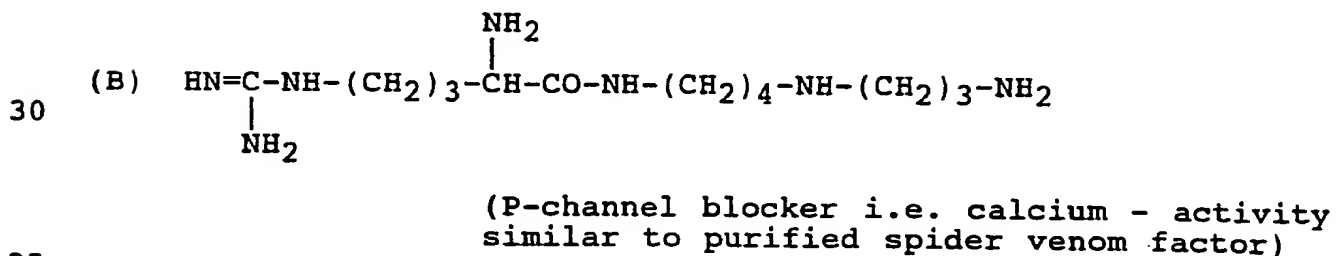
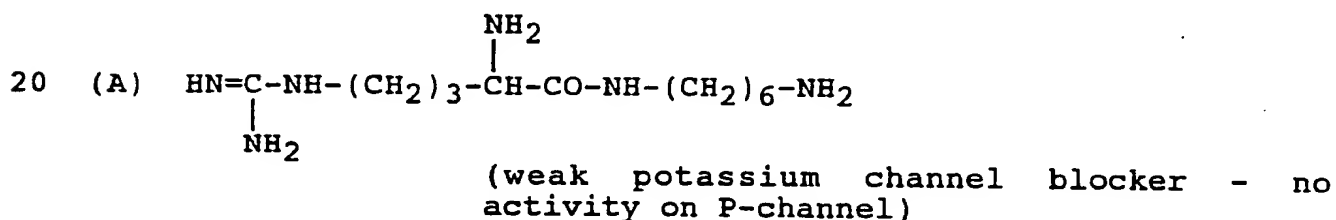
(c) On elemental analysis, the sample showed the relative proportions of C:N:H to be approximately 29:14:14.

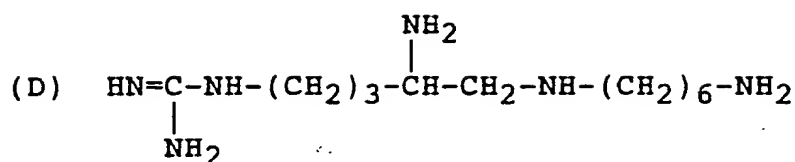
The conclusion drawn from this work was that the A. aperta P-channel blocker (hereafter sometimes referred to as "FTX") comprises a nonaromatic polyamine structure.

In accordance with the present invention, straight or branched chain nonaromatic polyamines have the ability to block or to enhance P-type calcium channels (i.e. channels that display one or more of the characteristics associated with the P-channels first identified in Purkinje cells and described above).

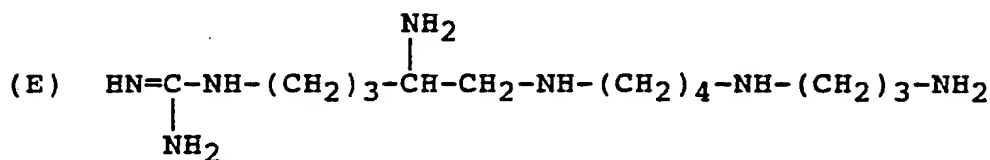
As used herein, "polyamine" will refer to a compound that has at least three -NH₂ and/or -NH- groups. Preferred are nonaromatic polyamines having an asymmetric methylene and/or nitrogen atom distribution, and most preferred are nonaromatic polyamines having a moiety bearing two or a plurality of amine groups on a single carbon atom or on neighboring carbon atoms. By asymmetric methylene and/or nitrogen atom distribution is meant that the two moieties of the compound are not the same: for example, spermine (which is not within the scope of this invention) has a symmetric distribution in that the nitrogen atoms are separated by four methylene groups on each side. Compound P, below, on the other hand has an asymmetric nitrogen atom distribution in that there is a higher number of N atoms (per number of carbon atoms) in the left moiety; compound H has an asymmetric methylene group distribution.

Illustrative of polyamines useful as ionic channel regulating agents are the following compounds:

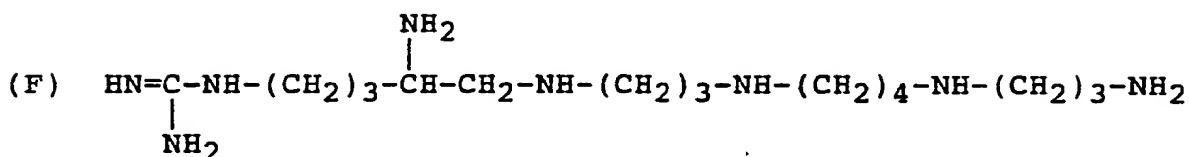




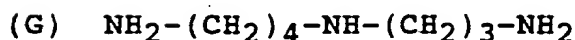
(potential potassium-channel activity)



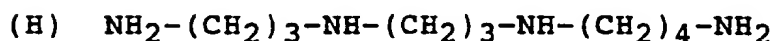
(P-channel blocker)



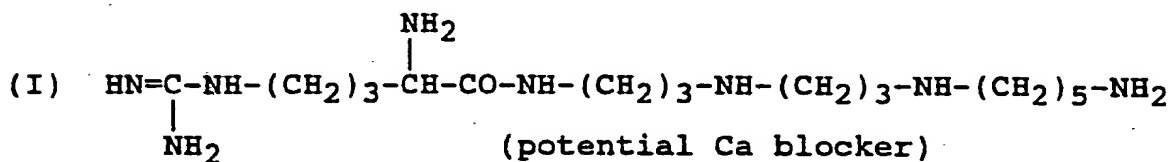
(potential Ca-channel blocker, inactive in P-channels)



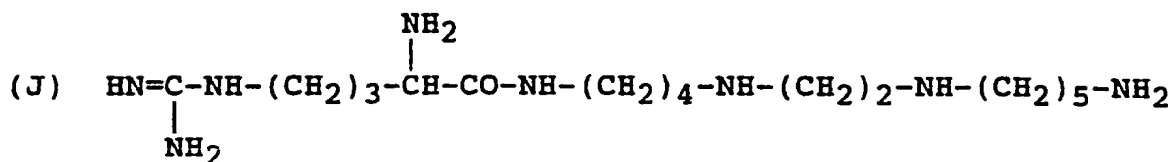
(weak P-channel blocker)



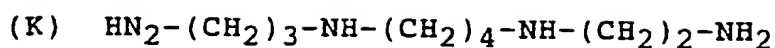
(not active in P-channels)



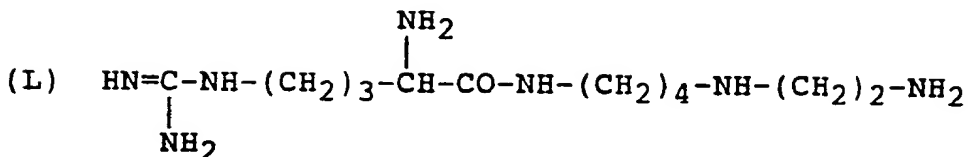
(potential Ca blocker)



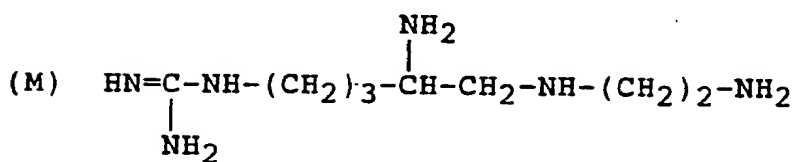
(potential calcium blocker)



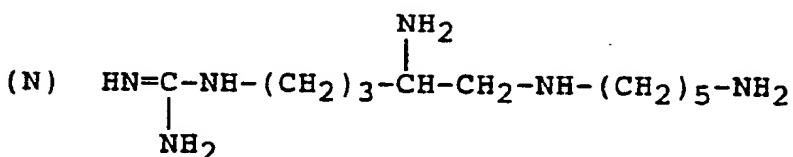
(potential calcium blocker)



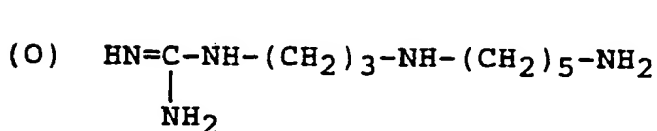
(potential P-channel blocker)



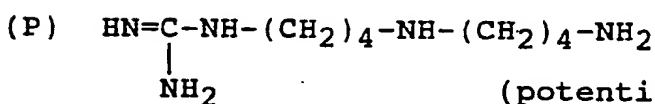
(potentially potassium-active)



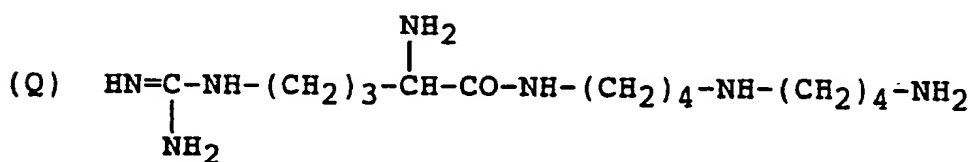
(potentially potassium-active)



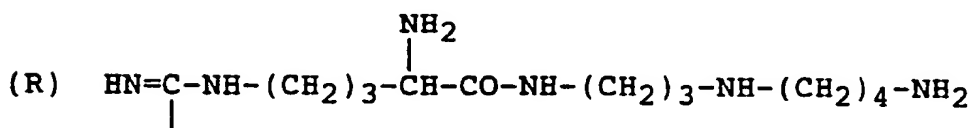
(potential sodium blocker)



(potential sodium blocker)



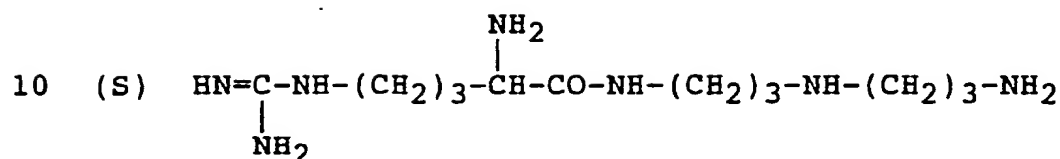
(P-channel blocker i.e., calcium - activity similar to FTX, purified spider venom factor)



NH₂

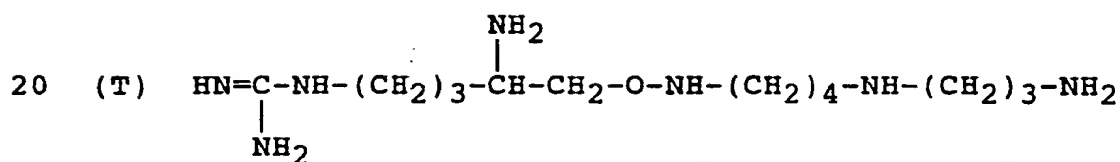
(P-channel blocker i.e., calcium
- activity similar to FTX,
purified spider venom factor)

5



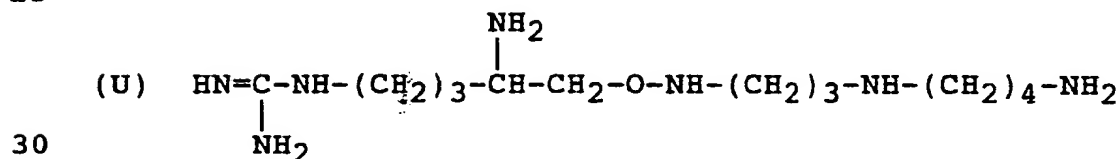
(P-channel blocker i.e., calcium
- activity similar to FTX,
purified spider venom factor)

15



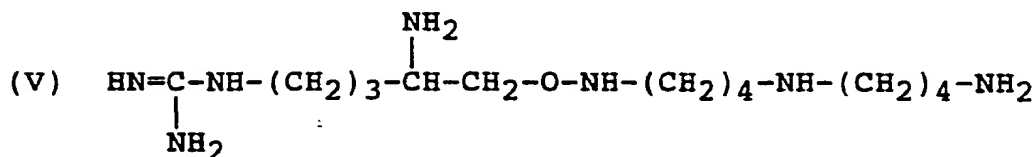
(P-channel blocker - FTX-like)

25



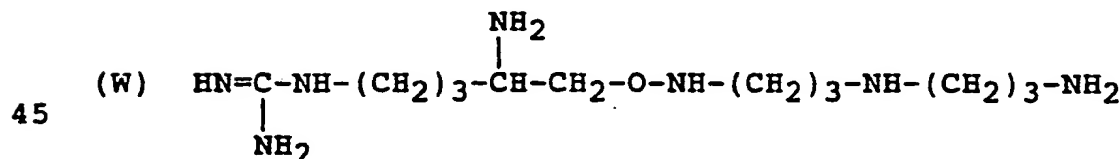
(P-channel blocker - FTX-like)

35



(P-channel blocker - FTX-like)

40



(P-channel blocker - FTX-like)

50

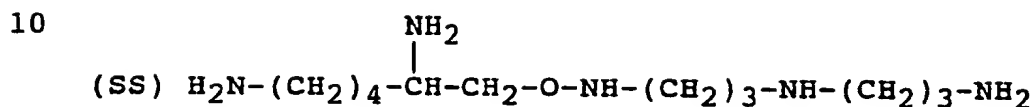
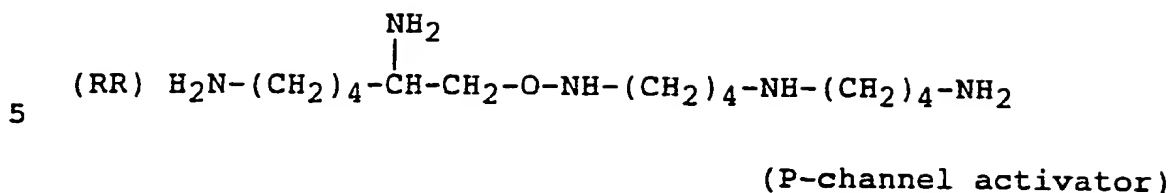
Examples of compounds having ionic channel blocking
and/or activating activity include derivatives or analogues of
compounds A-W having, .g., a dehydroxylated or decarboxylated

lysine moiety. In addition, many of the above compounds and their analogues are active on sodium or potassium channels.

Illustrative of polyamines useful as such channel regulating agents are the following compounds:

- 5
AA) $\text{H}_2\text{N}-(\text{CH}_2)_4-\underset{\text{NH}_2}{\text{CH}}-\text{CO}-\text{NH}-(\text{CH}_2)_6-\text{NH}_2$
(weak potassium blocker)
- 10
(BB) $\text{H}_2\text{N}-(\text{CH}_2)_4-\underset{\text{NH}_2}{\text{CH}}-\text{CO}-\text{NH}-(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_3-\text{NH}_2$
(P-channel activator)
- 15
(CC) $\text{H}_2\text{N}-(\text{CH}_2)_4-\underset{\text{NH}_2}{\text{CH}}-\text{CO}-\text{NH}-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_3-\text{NH}_2$
(does not block P-channels
20 potential P-channel activator)
- (DD) $\text{H}_2\text{N}-(\text{CH}_2)_4-\underset{\text{NH}_2}{\text{CH}}-\text{CH}_2-\text{NH}-(\text{CH}_2)_6-\text{NH}_2$
25 (potentially potassium active)
- 30 (EE) $\text{H}_2\text{N}-(\text{CH}_2)_4-\underset{\text{NH}_2}{\text{CH}}-\text{CH}_2-\text{NH}-(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_3-\text{NH}_2$
(potential calcium ac-
35 tivator)
- (FF) $\text{H}_2\text{N}-(\text{CH}_2)_4-\underset{\text{NH}_2}{\text{CH}}-\text{CH}_2-\text{NH}-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_3-\text{NH}_2$
40 (potential calcium ac-
tivator)
- (GG) $\text{H}_2\text{N}-(\text{CH}_2)_4-\underset{\text{NH}_2}{\text{CH}}-\text{CO}-\text{NH}-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_5-\text{NH}_2$
45 (potential calcium activator)
- (HH) $\text{H}_2\text{N}-(\text{CH}_2)_4-\underset{\text{NH}_2}{\text{CH}}-\text{CO}-\text{NH}-(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_2-\text{NH}-(\text{CH}_2)_5-\text{NH}_2$
50 (potential calcium activator)

- (II) $\text{H}_2\text{N}-(\text{CH}_2)_4-\underset{\text{NH}_2}{\text{CH}}-\text{CO}-\text{NH}-(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_2-\text{NH}_2$
5 (potential calcium activator)
- (JJ) $\text{H}_2\text{N}-(\text{CH}_2)_4-\underset{\text{NH}_2}{\text{CH}}-\text{CH}_2-\text{NH}-(\text{CH}_2)_2-\text{NH}_2$
10 (potentially potassium active)
- (KK) $\text{H}_2\text{N}-(\text{CH}_2)_4-\underset{\text{NH}_2}{\text{CH}}-\text{CH}_2-\text{NH}-(\text{CH}_2)_5-\text{NH}_2$
15 (potentially potassium active)
- (LL) $\text{H}_2\text{N}-(\text{CH}_2)_4-\underset{\text{NH}_2}{\text{CH}}-\text{CH}_2-\text{NH}-(\text{CH}_2)_5-\text{NH}_2$
20 (potentially potassium active)
- (MM) $\text{H}_2\text{N}-(\text{CH}_2)_4-\underset{\text{NH}_2}{\text{CH}}-\text{CO}-\text{NH}-(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_4-\text{NH}_2$
25 (P-channel activator)
- (NN) $\text{H}_2\text{N}-(\text{CH}_2)_4-\underset{\text{NH}_2}{\text{CH}}-\text{CO}-\text{NH}-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_3-\text{NH}_2$
30 (P-channel activator)
- (OO) $\text{H}_2\text{N}-(\text{CH}_2)_4-\underset{\text{NH}_2}{\text{CH}}-\text{CO}-\text{NH}-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_4-\text{NH}_2$
35 (P-channel activator)
- (PP) $\text{H}_2\text{N}-(\text{CH}_2)_4-\underset{\text{NH}_2}{\text{CH}}-\text{CH}_2-\text{O}-\text{NH}-(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_3-\text{NH}_2$
40 (P-channel activator)
- (QQ) $\text{H}_2\text{N}-(\text{CH}_2)_4-\underset{\text{NH}_2}{\text{CH}}-\text{CH}_2-\text{O}-\text{NH}-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_4-\text{NH}_2$
45 (P-channel activator)
- 50 (P-channel activator)



15 (P-channel activator)
In general, ion-channel blocking, modulating or activating polyamines useful in the present invention may be deemed to be encompassed by the formula



20 wherein y is an integer from 1 to 15, preferably from 1 to 6; x is an integer from 0 to 15, preferably from 0 to 6; and R is a nonaromatic organic group that contains at least one amine (including but not limited to amino, imino, amido or imido, aminoalkyl, amidoalkyl, imidoalkyl, etc; R may also be a
25 combination of two or more of the aforementioned groups) or may be appended to the remainder of the formula via the group -CX₂-O-NH-, wherein X is hydrogen or one of the X's is NH₂, with the provisos that (i) the molecule of the polyamine of the present invention is not symmetric in its distribution of
30 methylene and N-containing groups, and (ii) that it has at least three nitrogen atoms.

Preferably the polyamines of the present invention have a molecular weight below 800, and most preferably between 200 and 400.

35 With the understanding that the following statements constitute a generalization, which naturally will have some exceptions, it can be said that compounds of the above formula wherein x is 0 and R is not itself connected to the left NH group via a methylene or a methylene chain (i.e. R itself does
40 not help form an -N-C...-N-C-...N- configuration), will be potassium channel blockers, which preferably have y greater than 6 and most preferably simultaneously have R be an arginine

(as opposed to a lysine) moiety. Thus, for example, Arg-NH-(CH₂)₁₀-NH₂ is a potassium channel blocker and so is Arg-NH-(CH₂)₁₂-NH₂. The corresponding compounds with a lysine moiety at left also display the same potassium-blocking activity but are less active.

The compounds in which R is linked to the polyamine chain via a alpha-aminomethylene group (with or without an intervening -CO-, or -O- or -CH₂- group) and which have both x and y be positive integers within the above definition have calcium channel activity. In P-channels, this activity will be blocking activity if the left-hand moiety is arginine-based and calcium-activating activity if the left-hand moiety is lysine-based. For example, Compound B is a calcium (P-channel) blocker; Compound BB on the other hand is a calcium (P-channel) activator.

Compounds which do not have alpha aminomethylene group and which have a straight-chain polyamine portion with the configuration -NH-(CH₂)_x-NH-(CH₂)_y-NH₂ wherein both x and y are greater than zero will have sodium-blocking activity, the specificity of which to sodium channel appears to be "inversely proportional" to the number of methylene groups in the R moiety. Thus, H₂N-C(NH)-NH-(CH₂)₄-CO-NH-(CH₂)_x-NH-(CH₂)_y-NH₂ wherein x,y are e.g., 3,4; 3,3; 4,3; or 4,4 do not block calcium in P-channels but instead are weak sodium-channel blockers. As the number of straight-chain methylenes decreases from 4 to 3 the sodium-blocking activity increases, until finally when there are only two or fewer methylenes specificity decreases and both sodium and calcium are blocked simultaneously.

The polyamine compounds used in the present invention can be synthesized using well-known and commercially available starting materials and synthetic schemes well-known in the art. Alternatively, polyamine compounds within the scope of the present invention may be obtained from commercial sources.

For example, decarboxylated arginine (agmatine), or arginine ethyl ester, decarboxylated lysine or lysine methyl or ethyl ester can be purchased from Sigma Chemical Co., St.

Louis, MO. Other well known polyamines such as spermine, spermidine, 1,6 diaminohehexane, putrescine, cadaverine can also be obtained from Sigma or other commercial sources. Those which are not themselves active (e.g., spermine) can be used to
5 synthesize active compounds as follows:

One protocol involves using an amine ester in aqueous solution and reacting it under basic conditions with an appropriate (usually equimolar) amount of a diamine or other polyamine also in aqueous solution (or in liquid form).

10 See, generally, March, J., Advanced Organic Chemistry, 3d. Ed., 1985, Wiley & Son, New York.

Other synthesis schemes for compounds within the scope of the present invention are disclosed in Eldefrawi, A.T., et al., PNAS, 1988, 85:4910-4913; Hashimoto, Y., et al.,
15 Tetrahedron Letters, 1987, 28:3511-3514; and Yamamoto, H., et al., J. Am. Chem. Soc., 1981, 103:6133-6135 all of which can be employed with only such modifications, if any, as are readily apparent to those of ordinary skill in the art. Additional synthetic schemes may be devised based on International
20 Application WO89/07098 published August 10, 1989.

Another useful synthetic scheme which can be applied to the present compounds is the one disclosed in Melchiorre, et al., 1989, supra which involves use of a dicarboxylic acid to increase the length of a polyamine (in the presence of
25 $\text{CH}_3\text{CH}_2\text{OCOC}$ l, triethylamine and dioxane with addition of HCl and ethanol, followed by reduction in the presence of boron hydride, dimethyl sulfide and diglyme).

One preferred synthetic scheme for compounds within the scope of this invention that contain a decarboxylated arginine moiety (for example $\text{H}_2\text{N}-(\text{CH}_2)_3-\text{CH}(\text{NH}_2)-\text{CH}_2-\text{NH}-(\text{CH}_2)_3-$
30 $\text{NH}-(\text{CH}_2)_4-\text{NH}_2$ or $\text{H}_2\text{N}-(\text{CH}_2)_3-\text{CH}(\text{NH}_2)-\text{CH}_2-\text{NH}-(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_3-\text{NH}_2$) involves reducing the appropriate protected amine ester, e.g., $\text{Z}-\text{NH}-(\text{CH}_2)_4-\text{CH}(\text{NH}-\text{Z})-\text{CO}-\text{OEt}$, (wherein Z is an appropriate protective group such as $\text{Ph}-\text{CH}_2-\text{O}-\text{CO}-$ wherein Ph is phenyl) in
35 the presence of THF (at -78 degrees C) and di-isobutylaluminum hydride to the corresponding aldehyde, aminating this product in the presence of sodium cyanoborohydride to produce the

desired polyamine which can then be deprotected and purified according to well-known methods.

Alternatively, the protected amino acid (e.g. the free acid corresponding to the ethyl ester referred to above) can be first converted to the corresponding mixed anhydride by reacting with, e.g., Et-O-CO-Cl (ethoxycarbonyl chloride) and TEA (triethylamine) and THF (tetrahydrofuran) at -20 degrees C. The anhydride can then be reacted with an alcohol, e.g., methanol at -10 degrees C in the presence of sodium borohydride to convert the mixed anhydride to the corresponding alcohol. The alcohol can then be converted to the corresponding halide according to well-known halogenation techniques. The halide can be condensed with the appropriate amine (in the presence of an acid scavenger such as K₂CO₃ or dimethyl formamide) to yield the target protected compound which can be deprotected by e.g., hydrogenolysis (H₂ palladium/carbon 10%) or where the protecting group is Boc, with trifluoroacetic acid to yield the actual target compound.

Compounds within the invention in which R is linked to the NH group of the -N-C-..N chain via an -O- linkage can also be synthesized via well-known techniques. For example, the appropriate amino acid (e.g., arginine) can be reacted preferably with reflux with the appropriate straight-chain polyamine, e.g., spermine, or H₂N-(CH₂)₄-NH-(CH₂)₄-NH₂, or H₂N-(CH₂)₃-NH-(CH₂)₃-NH₂, in the presence of an acid (e.g., acetic acid) and a peroxide (such as hydrogen peroxide) to yield the desired compound in accordance with the invention (in the present example, this compound could be depicted by the formula T-CX₂-O-NH-(CH₂)_x-NH-(CH₂)_y-NH₂ wherein T-CX₂-O- would be a modified arginine moiety with X being H (or one of the two X being NH₂).

Compounds within the formula I wherein R does not have an alpha amino group appended to a -CH- group and their isomers (e.g., HN=C(NH₂)-NH-(CH₂)₄-NH-(CH₂)₄-NH-(CH₂)₃-NH₂) can be synthesized from the appropriate polyamine (for example, H₂N-(CH₂)₄-NH-(CH₂)₄-NH-(CH₂)₃-NH₂) using a guanylation agent

such as 3,5-dimethyl-1-guanyl pyrazole nitrate under ethanol reflux. The target compound can be isolated as the nitrate.

When used as laboratory reagents the compounds of the present invention can be used in widely ranging units from nanomolar to no particular upper limit. It will be appreciated that the amount that needs to be used in each instance will depend on the activity of a particular compound (whether the compound is a potent blocker, activator, or modulator), on the sensitivity of the specific channel on which this compound is active (whether the channel properties are easily modifiable), on the specificity of the activity of the particular compound (whether the compound acts exclusively on one type of channel) and on other factors which are well-recognized in the art to be subject to optimization. Such optimization can be easily achieved without undue experimentation by well-known methods.

When the compounds of the present invention, including pharmaceutically acceptable salts thereof are used for pharmaceutical purposes (for example to bring about the behavioral modifications or to treat the behavioral alternatives referred to in the Background section, above), they may be incorporated in pharmaceutical compositions in oral, enteral, topical, depot, or parenteral dosage forms containing one or more of the present compounds, in association with one or more pharmaceutically acceptable excipients, fillers, salts, coatings, carriers or diluents such as are customarily used with pharmaceutical preparations, e.g., tablets, sustained-release preparations, gelatin capsules, injectable solutions, suppositories, or coupled to a drug delivery system, etc. The active ingredient dosage ranges will encompass the following preferred ranges: 10-100 mg/kg of a particular compound or compounds in rodents, and 0.05-50 mg/kg in man. It will of course be preferred to use the minimum amount which will accomplish the desired effect in order to avoid as many side effects as possible. Moreover, the unit content of each dosage form need not by itself constitute an effective amount of active ingredient since a plurality of dosage forms may be administered to achieve an effective amount in combination. In

addition, the amount used and the duration of the treatment will also be subject to optimization and will vary depending on the severity and responsiveness of the condition to be treated, the age, weight, and physical condition of the patient, and often also on the administration route.

The invention is further described below by reference to specific Examples which are intended to illustrate it without limiting its scope:

EXAMPLE 1: ASSAYS FOR P-CHANNEL BLOCKING ACTIVITY

A. Purkinje Cell System

Adult Hartley guinea pigs (400-600 grams from Camm Research Institute, Wayne, New Jersey) were decapitated with a small animal guillotine under ether or sodium pentobarbital (Abbott Pharmaceuticals, Inc., N.Chicago, Ill., 40 mg/kg i.p.) anesthesia. A rapid craniotomy was performed to remove the squamous portion of the occipital bone, which allowed the total cerebellar mass, including the cerebellar nuclei, to be detached quickly with a metal spatula. The tissue was then immediately immersed in aerated Krebs-Ringer solution containing 124mM NaCl; 55mM KCl; 1.2mM KH_2PO_4 ; 2.4mM CaCl_2 ; 1.3mM MgSO_4 ; NaHCO_3 (26mM); and 10mM glucose. This solution was kept refrigerated at 6°C. The cerebellar mass was then transacted sagittally and a single cell slice about 2mm thick was isolated from the vermis or from one of the hemispheres. The slice was affixed with cyanoacrylate to the bottom of a plexiglass cutting chamber and agar blocks were used to surround the slice, thus providing side support. Once secured, the tissue was immersed in Krebs-Ringer solution at 6°C and further sectioned with an Oxford G501 Vibratome (Ted Pella, Inc., Tustin, CA) to yield about six 200-(or 300-)micron thick cerebellar slices, containing sagittal sections of all the cerebellar folia in a given rostrocaudal plane as well as central white matter and cerebellar nuclear cells. Following this procedure, the slices were incubated in oxygenated (95% O_2 ; 5% CO_2) Krebs-Ringer solution at 37°C for about one hour.

After incubation, a slice was transferred to a recording dish such as that described in Llinas, R. et al, J.

Physiol., 305:171, 1980. The cerebellar slice was placed in a Sylgard plate (Corning Glass, Corning, New York) at the bottom of the recording chamber and secured with a bipolar stimulating electrode pressing lightly on the white matter. The experiments were conducted at a chamber temperature of 37°C maintained by a surrounding temperature-controlled water bath. The saline (Ringer's) solution used for continuous perfusion was also kept at 37°C.

Various channel blocking agents were used to block conductances under study or to block ionic conductances that would interfere with a particular experiment: Tetrodotoxin ("TTX" 10^{-6} M) was used to block sodium conductance; triethylammonium chloride ("TEA", 30mM), was used to block potassium conductance; nitrendipine (10^{-5} - 10^{-6} M) was used to block the L-channel.

Various preparations of compounds to be tested for P-channel blocking activity were introduced in the bath and the flow was turned off for various time periods. These preparations were added in aliquots of 20-40 microliters from a solution containing a particular molar concentration of each substance to be tested. The volume of the extracellular bath was 4 cc.

Purkinje cells were impaled with recording micropipettes under direct vision using Hoffman modulation microscopy (Hoffman, R., J. Microsc. 110:205-222, 1977). Intracellular recordings were obtained with micropipettes filled with 3M potassium acetate or 1M tetraethylammonium chloride (TEA), and having an average D.C. resistance of 60-80 megohms. Synaptic activation of the cells was effected with a bipolar stimulation electrode located on the white matter at the basis of the folium studied. Direct stimulation of the Purkinje cells was implemented with a high-input impedance (10^{12} Ohms) bridge amplifier.

In this series of experiments, various concentrations of the compound being tested were used as specified below: The smaller the amount of active compound that achieves blockage the higher the affinity of the active polyamin s for calcium

channels. Representative results of the experiments described below are illustrated in Figure 1.

Before the introduction of a polyamine or other channel blocking compounds in the bath, upon injection of an outward (depolarizing) current pulse, the neurons responded with firing having both sodium-dependent and calcium-dependent spikes in accordance with the normal electrical response of the Purkinje cells in the absence of a P-channel blocker (Fig. 1A). The cell responses were also measured at several time intervals after introduction of a polyamine compound in the recording chamber medium. Typically, in the presence of a P-channel blocker, a small depolarizing current (approximately 0.3 or 0.45 nA for 62 msec) generates a burst of potential spikes and a plateau potential, the latter due to the non-inactivating ("persistent") sodium conductance; calcium spikes are substantially reduced or extinguished and there is no calcium-dependent component to the plateau potential (Fig. 1B). Figure 1B was recorded after the addition of spermidine to a final concentration of 0.8mM.

The apparent absence of the sodium-dependent fast spikes in Fig. 1B that would be expected to follow the first such spikes observed is due to the substantially increased resistance of the cell (indicated by the sodium-dependent plateau potential). Blockage of calcium channels precludes calcium ions from entering the cell and consequently the exit from the cell of potassium ions is not activated. The results in Figure 1B are comparable to addition of the P-channel blocker isolated from funnel-web spider venom, e.g., A. aperta, as disclosed in U.S. Pat. Appln Ser. No. 219,905.

In Figure 1C (upper trace), potassium conductance was blocked with TEA which was also added to the bath (which already contained TTX and spermidine). The remaining calcium spike is due to the existence of another type of calcium channel in Purkinje cells. (The P-channel has been blocked by spermidine as in the experiment of Fig. 1B). The spike of Fig. 1C is due to a calcium conductance that is polyamine-resistant (and funnel-web venom resistant) and is therefore not due to

the P-channel. This remaining calcium conductance is blocked by the addition of dihydropyridine (Fig. 1C, lower trace) and is also blocked by the addition (to the same preparation, 1D upper trace) of antibiotics such as streptomycin (Fig. 1D, lower trace).

The same experiment can be performed using compounds within the present invention that are activators. Of course, in that case, the result will be an increase in the rate of rise of the calcium spike which could be recorded in an experiment such as the one in which Fig. 1 was generated (except that no blocking agent will be added).

B. Electrophysiology with Squid Stellate Ganglia

In squid synapse voltage clamp and current clamp experiments, the following observations were made:

Action potentials were induced by direct electrical stimulation of the presynaptic nerve bundle of the giant synapse in squid stellate ganglia. The thus evoked postsynaptic action potential is shown in Figure 4 in the absence of any calcium-blocking agents in the bath (trace A). After addition in the 3 cc bath of 0.15 ml of the preparation of compound E (described below), the postsynaptic action potential is markedly reduced (trace B) four minutes after such addition and completely extinguished at 6 minutes.

The decrease and eventual extinction of the postsynaptic action potential is attributed to blockage by the P-channel blocking factor (here compound E) of the presynaptic calcium channels. That calcium channels are involved was demonstrated in another type of voltage clamp experiment (Fig. 6) in which the postsynaptic potential in squid synapse (in a preparation containing tetrodotoxin and 3-aminopyridine) is reduced over time after the addition of 0.4 ml of a 1mM solution of the P-channel blocker Compound B in the 3 cc bath. In Figure 6, the squid postsynaptic action potential evoked by a 38.2 mV voltage clamp for 10 msec from a resting potential of -60mV was recorded 1, 6, 10 and 13 minutes after addition of the P-channel blocker. The potential was reduced and eventually extinguished. It should be noted that squid synapse is less

accessible to reagents than Purkinje cells; therefore greater amounts of a blocking agent and/or a longer waiting time are necessary for a particular effect to be observed.

Figure 5 shows yet another voltage clamp experiment in which a 28mV/5.5 msec voltage step is applied to the presynaptic terminal (trace C) in a squid synapse preparation, wherein sodium conductance was blocked with TTX and potassium conductance was blocked with 3-aminopyridine. The applied step voltage generates a compensating inward ionic current in the presynaptic cell (trace B) which is due solely to calcium since the other conductances are blocked. At the postsynaptic cell a postsynaptic potential (EPSP) is evoked, which is the normal response (upper curve, trace A) in the absence of a P-channel blocker (here Compound B). At the same time, in the presence of Compound B, the presynaptic ionic current is also reduced (trace B, upper curve, i.e. curve with the lesser amplitude).

This indicates that the P-channel blocker acts on the presynaptic calcium channels since the time relationship in Fig. 5 of the presynaptic calcium entry to the postsynaptic response remains constant and only the amplitude of the postsynaptic response is reduced in direct relationship to the reduction of the presynaptic calcium current. This means that the P-channel blocker blocks presynaptic calcium channels which impedes calcium influx and consequently the expected transmitter release does not occur. The reduced transmitter release in turn causes a reduced postsynaptic response.

In a current clamp experiment using squid synapse, a fixed amplitude depolarizing current (400 nA, 8.4 msec) is applied to the presynaptic terminal. In the presence of TTX and 3-aminopyridine, the presynaptic potential (calcium spike) is as depicted in Figure 7A, upper trace.

The corresponding postsynaptic potential (EPSP) is depicted in Figure 7B (upper trace). Seven minutes after addition of P-channel blocker (here Compound B) the presynaptic calcium spike is reduced (7A, lower trace) and so is the EPSP (7B, lower trace). The presynaptic calcium spike after blockage varies in amplitude only. The evoked postsynaptic

potential also varies in amplitude. Moreover, the time course of the EPSP after blockage is the same as in the absence of blocker. This indicates that the polyamine P-channel blocking agents act presynaptically and do not have a postsynaptic effect: if a postsynaptic blockade was present, the time course of the EPSP would also be different (in addition to its amplitude being lower).

Figures 2 and 3 demonstrate that the effect of the P-channel blocking agent on the presynaptic calcium current and postsynaptic potential is dose-dependent: both decrease in amplitude with an increasing amount of P-channel blocker used in the extracellular medium. (In Fig. 2 venom was used and in Fig. 3 partially purified A. aperta P-channel blocker was used). The partially purified preparation was processed by deproteination from venom (boiling), centrifugation, removal of the pellet, addition to original volume of water followed by twice butanol extraction in 20 volumes of butanol. The aqueous phase was used.

The results described herein are qualitatively the same regardless of the particular P-channel polyamine blocker used. the only difference is the potency of the particular blocker. Compounds A and B and especially compound E appeared to be much more potent than spermidine, and, for that reason, these compounds are preferred. Also preferred is the purified P-channel blocking factor from funnel-web spiders purified by deproteination followed by cation-exchange chromatography as described above.

Figure 8 is a superimposition of tracings from a voltage clamp experiment in squid (similar to that illustrated in Fig. 5, but with the background removed) in which a 35 mv/1 msec voltage step (trace V) is applied to the presynaptic terminal in a squid synapse preparation of the type described above, wherein sodium conductance was blocked with TTX and potassium conductance was blocked with TEA. In this particular preparation four presynaptic ionic current measurements were made after application of the same voltage step over time before (trace I-1) and 1, 2 and 5 minutes after application of

about 1 mg of the reaction product of lysine ester and spermidine from Example 2 per cc of bath (traces I-2 through I-4). Without this product, the amplitude of the ionic current would progressively decrease since the cells eventually die.

5 However, after application of compound BB, a tail current is generated (which is due solely to calcium since other conductances are blocked). The calcium current maximum amplitude first slightly decreases (I-2) and then progressively increases (I-3, I-4) and displays a progressively larger peak (starting

10 at 180 nA for I-2) which means that under the influence of the activating agent, the calcium channel open probability increases and the presynaptic cell receives more and more calcium from the extracellular medium. A small change in presynaptic calcium current normally (i.e. in a fresh cell preparation

15 without calcium blockers or activators) produces a large change in EPSP. EPSP measurements in this post-synaptic cell indeed showed a progressively higher amplitude for the EPSP (see corresponding EPSP tracings P-1 through P-4). This experiment also shows that the lysine-spermidine conjugate activates

20 calcium channels and acts presynaptically since the changes in the EPSP directly relate to the changes in the presynaptic cell. It is anticipated that the channel activated by this compound is specifically of the P-type, and that the activating effect will not subside by addition of blocking agents for

25 other calcium channel types. Also, in an experiment where the reaction product of lysine ethyl ester and spermidine (see Example 2) was placed in the bath, there was no calcium channel blockage after the subsequent introduction in the bath of the calcium channel blocking factor purified from A.aperta venom,

30 purified as described above.

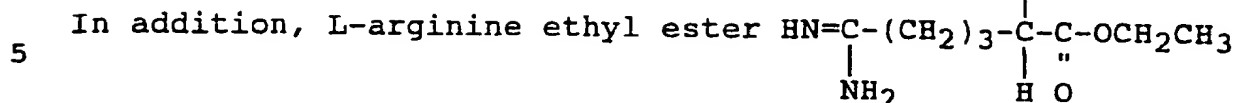
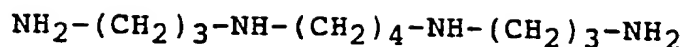
EXAMPLE 2: POLYAMINES USED FOR BLOCKING OR ACTIVATION

The following compounds were purchased or synthesized and tested for P-channel blocking activity and specificity of such activity:

35 The following were obtained from Sigma:

spermidine	$\text{NH}_2-(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_3-\text{NH}_2$
1,6 diaminoheptane	$\text{NH}_2-(\text{CH}_2)_6-\text{NH}_2$

spermine



also from Sigma was used to synthesize compounds B and A, above. Briefly, the synthesis of Compound B involved use of 3×10^{-3} moles of L-arginine ethyl ester which was dissolved in 5ml of 1N NaOH. An equimolar amount of spermidine was added dropwise and the reaction was allowed to proceed at 25°C, pH 14 under stirring. The product was brought to pH 7.4 by addition of 5N HCl. Forty-microliter aliquots of this preparation of Compound B were used in experiments with the assay system of Example 1.

Compound A was synthesized in the same manner except that an equimolar amount of $\text{NH}_2-(\text{CH}_2)_6-\text{NH}_2$ was first dissolved in a minimum volume of water before adding to the arginine ester in 1N NaOH.

Compound E and its isomer was synthesized as described above.

In addition, several antibiotics containing polyamine moieties were obtained commercially and tested. These were:

Gentamycin (from Elkins-Sinn, Inc., Cherry Hill, N.J.).

Streptomycin (Pfipharmecs, a division of Pfizer, Inc., New York, N.Y.).

Vancomycin (Eli Lilly Industries, Inc., Carolina, Puerto Rico).

It is anticipated that other antibiotics containing amine groups such as Kanamycin will have the same activities, any differences being of degree rather than kind. Several such antibiotics are commercially available.

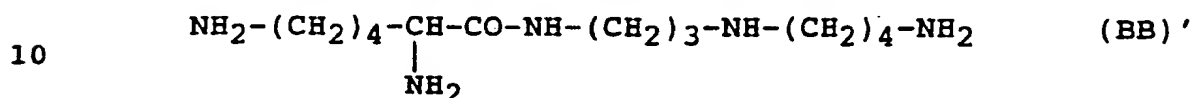
Each of the three antibiotics listed above as having been tested was dissolved in water and 0.1, 0.4, and 0.8 mM amounts were used. 1 mM of vancomycin was used.

Finally, the compounds 4-(3-aminopropyl) morpholine from Aldrich Chemicals Milwaukee, Wisc., and PHTX433

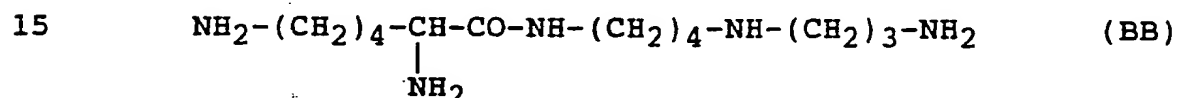
("PTX433"), a compound described in Eldefrawi et al., 1988, supra (believed to be a glutamate receptor antagonist for locust leg muscle) were also tested.

Amounts of up to 1.0 mM of the morpholine derivative and even larger amounts of PTX433 were used for electrophysiological evaluation of their P-channel blocking activity.

The P-calcium channel activating compounds



and



were synthesized by reacting an equimolar mixture of lysine and spermidine in water, in the presence of an equimolar amount of a water-soluble carbodiimide namely N-cyclohexyl-N'-[(2-morpholinyl)-ethyl]-carbodiimide-methyl-p-toluene sulfonate (Aldrich Chemical Co.) at pH 4.7. The reaction mixture was allowed to remain 20 hours at room temperature under stirring. The product mixture was extracted three times with ethyl acetate (total about 10 volumes) and the aqueous phase was recovered and brought up to pH 7.2. The aqueous phase can be further purified by FPLC (high performance liquid chromatography-Pharmacia) under a 0-1.0 NaCl gradient, pH 7.5 and a flow rate of 1ml/min using a cation-exchange column such as Mono-S (5x50 mm) from Pharmacia. Alternatively, another conventional further purification method can be used. However, the aqueous phase resulting from the above-described final ethyl acetate extraction can be used as is.

Although the foregoing synthesis method does not differentiate between the two compounds (BB) and (BB)' and in all likelihood yields a mixture of these compounds, the calcium channel activating potency of the extracted product (see Example 1) indicates that most probably both are active.

The foregoing technique can be used to synthesize other lysine-based polyamines within th Formula I.

Another synthetic technique that can be used to yield only compound (BB)' is to first react the lysine with 1,3 diamino propane using, e.g., the above carbodiimide method, and then reacting the product (preferably) after purification with 1,4 diamine butane using one of the methods described above for the compounds of Formula I. The order of use of the diamines can be reversed for synthesizing the (BB) product.

EXAMPLE 3: FURTHER STUDIES WITH CALCIUM ACTIVATOR

In single channel studies conducted as described in PCT Application PCTUS89/00558 but using the product of lysine-spermidine reaction the foregoing calcium channel activator increased the open probability of the calcium channels but did not appear to alter the conductance of individual channels.

The calcium blocking factor isolated from A.aperta venom or compound B or E are lethal to rats when administered at a dose of 50 to 100 microliters. Similar amounts of purified compound BB do not kill these animals. On observation, rats are placid but display no visible movement disorders, and do not extend their limbs when picked up. Compound BB (and/or BB') appeared to have a calming effect similar to a tranquilizing effect but without much muscle relaxation.

EXAMPLE 4: FLUORESCEIN-LABELLING OF CALCIUM-REGULATING AGENTS

Arginine was labelled by mixing with an equimolar amount of fluorescein in sodium borate buffer, pH 9.5 and allowing the mixture to react overnight in the dark under stirring. Excess fluorescein was extracted by washing three times with 10 volumes ethyl acetate per wash. The thus labelled arginine can be used to synthesize compounds of Formula I. Lysine ethyl ester can be similarly labelled. Thus, labelled Formula I compounds (both blockers and activators) can be used in fluorescence experiments, using methods well-known in the art.

EXAMPLE 5: RESULTS OF FURTHER ELECTROPHYSIOLOGICAL STUDIES

P-channel activity was recorded in the system of Example 1 in the absence and presence of various amounts of the compounds listed in Example 2.

The results for the P-channel were as follows:

Spermine and 1,6 diaminohehexane did not block the P-channel at the concentrations tested (up to about 1 mM). The calcium conductances attributable to P-channels were the same in the presence and in the absence of these compounds. By contrast, spermidine blocked the P-channel selectively but not with high potency (compared to the potency of spider venom or purified P-channel blockers from venom).

Spermidine has no effect on the L-channel up to a concentration of 800 micromolar. 4-(3-aminopropyl)morpholine did not block the P-channel up to a concentration of 1 mM. PHTX433 did not block the P-channel up to a concentration of 10 mM in Purkinje cells, but blocked the postsynaptic response in squid (about 300 micromolar).

By contrast, Compounds A, B and E were potent P-channel blockers producing complete P-channel blockage at concentrations lower than 0.5 mM. None blocked the dihydropyridine-sensitive L-channel at the concentrations tested (well below 1 mM). The other channels (sodium, potassium) were not affected. The minimum effective concentrations of the P-channel blocking agents of this invention are micromolar or less; this can be determined by routine experimentation using serial dilutions of these agents. Preferred are concentrations up to 1mM but it should be appreciated that the choice of concentration of each agent is subject to optimization as is well-known in the art and also depends on whether partial or total P-channel blockage is desired.

The antibiotics block both the sodium channel at concentrations below 1 mM as well as the L-channel. None of the antibiotics tested block the P-channel at concentrations below 1 mM although P-channel blockage is seen (as well as L- and sodium channel blockage) when the concentration of the aliquot introduced in the bath is higher than 10 mM. In no instance was P-channel blockage by the antibiotics potent or specific, consistent with the presence of aromatic rings in all the antibiotic molecules tested and despite the presence of the

moiety $\text{HN}=\text{C}(\text{NH}_2)-\text{NH}-$ possessed by arginine in streptomycin and of various amine groups in the other antibiotics.

These results indicate that the nonaromatic blocking polyamines of the present invention block calcium channels of the P-type specifically (i.e., without affecting sodium, potassium or L-type calcium channels). In particular, these agents block presynaptic calcium channels and thereby control transmitter release from the presynaptic to the postsynaptic neuron. Analogous specificity is expected of the activating agents of the present invention.

Various other compounds can be obtained and/or tested as described above. It is anticipated that the results will demonstrate the general applicability of the following observations:

- 15 - The presence of aromatic rings (including heterocycles) inhibits P-channel regulating ability of a polyamine compound, and may inhibit specificity.
- 20 - The presence of three amino groups is essential for activity, provided that the polyamine is not symmetrical in methylene/amine group distribution.
- 25 - Activity increases with increasing nitrogen content of a polyamine (everything else being equal).

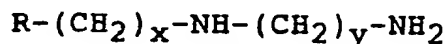
The P-channel activating compounds of the present invention differ from the arginine-based blockers only in that arginine has been replaced by lysine. It can therefore be seen that polyamines can have different and in fact opposing effects on P-type channels depending on the structure of the group R. In fact, it is anticipated that some calcium channel activators (or blockers) would be specific to P-channels from cells of a particular animal species and could therefore serve as markers of additional distinguishing characteristics of subtypes of P-channels.

EXAMPLE 6:

The compound BB was administered to a rat in amounts ranging between 50 and 100 mg/kg. The rat was subsequently
5 injected with 40mg/kg (actual volume: 0.2 ml) of sodium phenobarbital (NEMBUTAL), a dose which normally produces total anesthesia, and in many cases death. The BB-injected rat by contrast, did not become unconscious upon injection. It took two additional doses of nembutal to anesthetize this rat. The
10 experiment was repeated except this time two rats were injected with compound BB, one with the same dose as was administered in the previous experiment and one with twice that dose. Immediately after injection, the first rat appeared normal and the second exhibited reduced activity on observation. After
15 each was injected with 40mg/kg of nembutal, the first rat showed no effect but the second appeared to wake up. Subsequent 40mg/kg injections of Nembutal caused the first rat to become anesthetized at a total dose of 160mg/kg but the second rat received a total of 200 mg/kg before he was anesthetized,
20 and received 240 mg/kg before he was killed. The normal LD-50 for Nembutal in rats is about 60 mg/kg. Therefore this dose is enormous by comparison. The implication is that compound BB which is a calcium-channel activator causes resistance to barbiturate action. Furthermore, it causes animals to be
25 serene without being tranquilized, in that it does not cause much muscle relaxation and has potential utility as a prototype drug for anxiolysis.

WHAT IS CLAIMED IS:

1 1. A method for regulating calcium ion transport
 2 across cellular membranes possessing calcium channels that are
 3 dihydropyridine-resistant, conotoxin-resistant and octanol
 4 resistant comprising exposing a cell membrane possessing said
 5 channels to a nonaromatic polyamine compound of the formula
 6 (I):



8 wherein y is an integer from 1 to 15;

9 x is an integer from 0 to 15;

10 R is a nonaromatic organic group containing at least
 11 one amino, imino, amido, imido and/or may be appended to
 12 the remainder of the formula via a $-CX_2-O-NH-$ group
 13 wherein X is hydrogen or one of X is NH_2 with the proviso
 14 that

15 the distribution of at least one of the methylene groups or
 16 nitrogen atoms about the molecule of said polyamine is asym-
 17 metric and that the compound contains at least three nitrogen
 18 atoms;

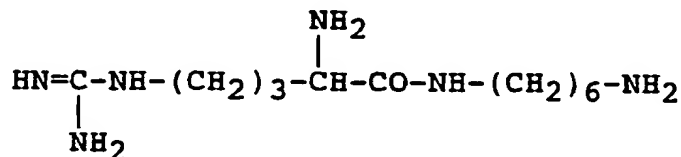
19 said polyamine being present in an amount effective to regulate
 20 conductance attributable to said channels.

1 2. The method of claim 1 wherein said polyamine
 2 compound has the formula I wherein x is 1 to 6 and y is 1 to 6.

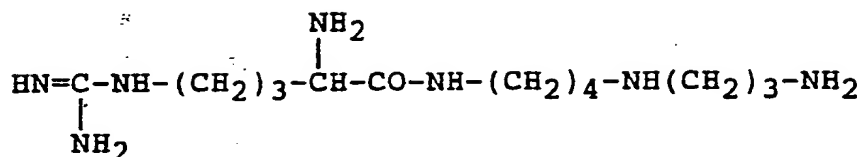
1 3. The method of claim 1 wherein said channels are
 2 P-channels.

1 4. The method of claim 2 wherein said channels are
 2 P-channels and said cells are mammalian central neurons.

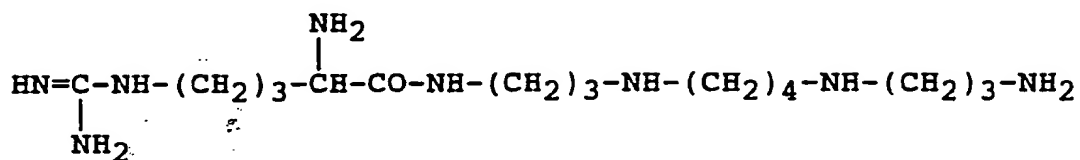
1 5. The method of claim 1 wherein said polyamine has
 2 the formula:



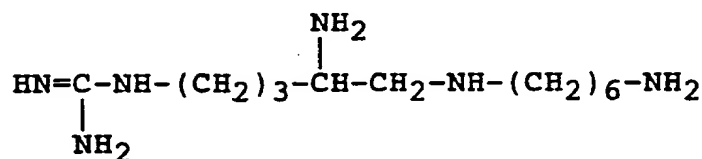
6. The method of claim 1 wherein said polyamine has the formula:



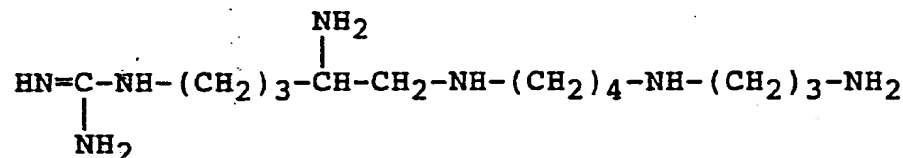
7. The method of claim 1 wherein said polyamine has the formula:



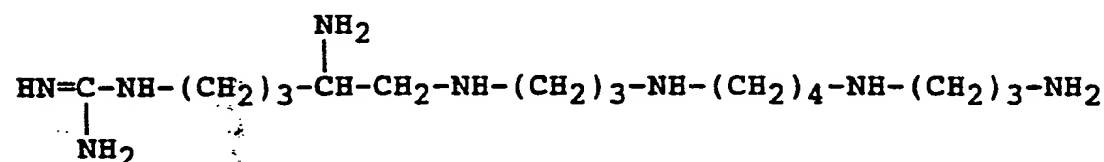
8. The method of claim 1 wherein said polyamine has the formula:



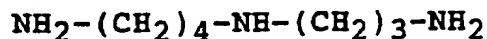
9. The method of claim 1 wherein said polyamine has the formula:



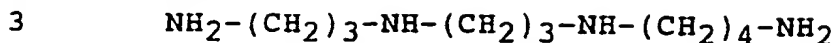
10. The method of claim 1 wherein said polyamine has the formula:



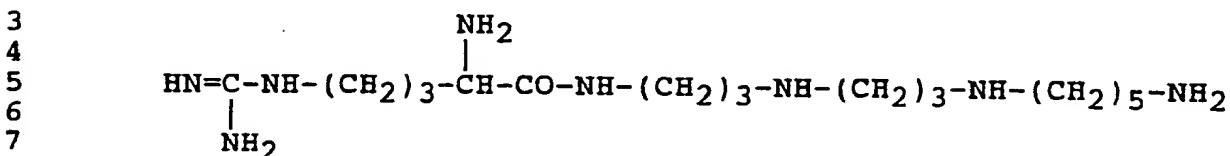
11. The method of claim 1 wherein said polyamine has the formula:



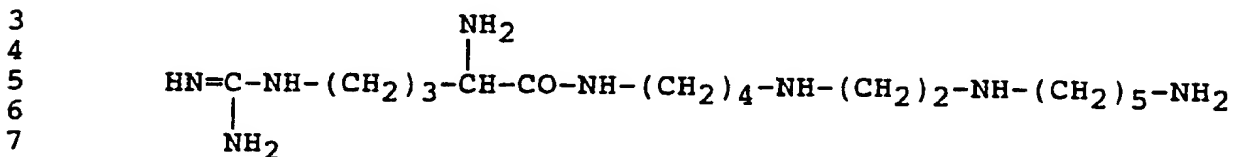
1 12. The method of claim 1 wherein said polyamine has
2 the formula:



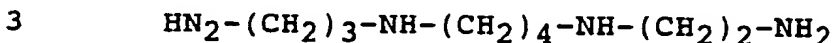
1 13. The method of claim 1 wherein said polyamine has
2 the formula:



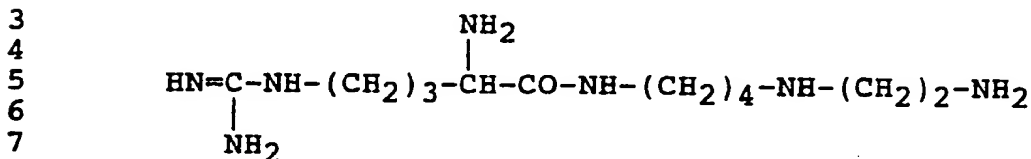
1 14. The method of claim 1 wherein said polyamine has
2 the formula:



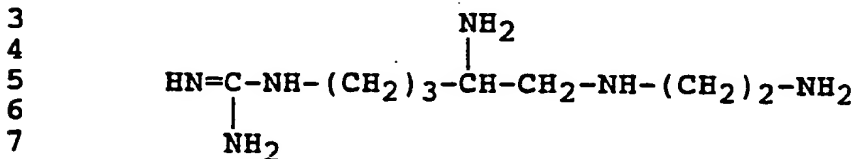
1 15. The method of claim 1 wherein said polyamine has
2 the formula:



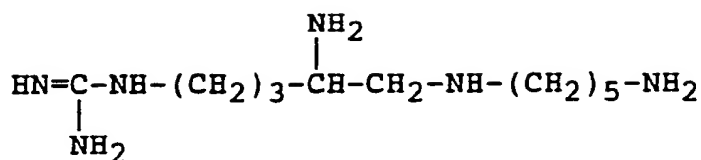
1 16. The method of claim 1 wherein said polyamine has
2 the formula:



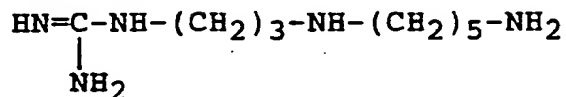
1 17. The method of claim 1 wherein said polyamine has
2 the formula:



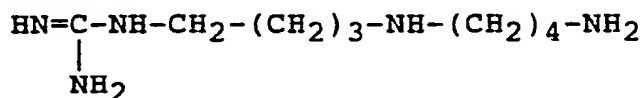
1 18. The method of claim 1 wherein said polyamine has
2 the formula:



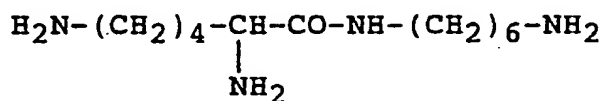
19. The method of claim 1 wherein said polyamine has the formula:



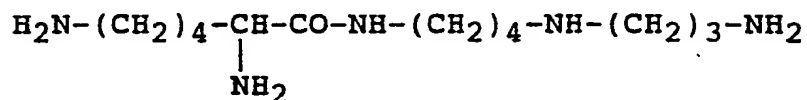
20. The method of claim 1 wherein said polyamine has the formula:



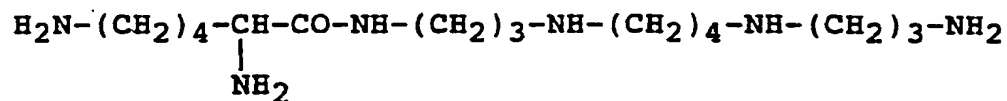
21. The method of claim 1 wherein said polyamine has the formula:



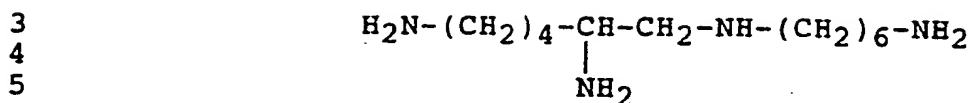
22. The method of claim 1 wherein said polyamine has the formula:



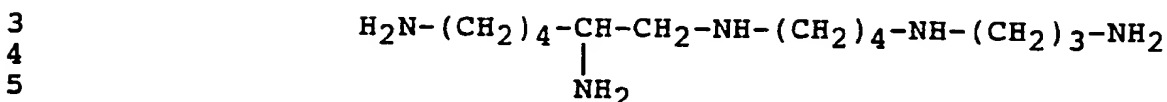
23. The method of claim 1 wherein said polyamine has the formula:



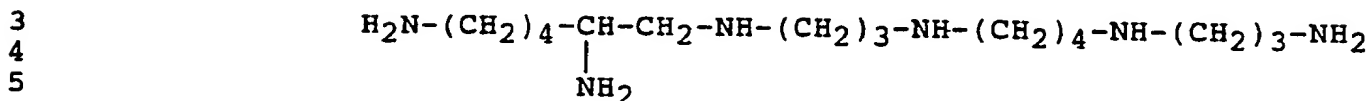
1 24. The method of claim 1 wherein said polyamine has
2 the formula:



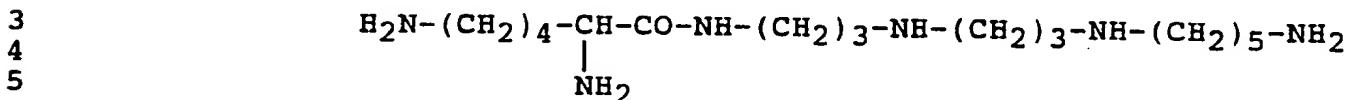
1 25. The method of claim 1 wherein said polyamine has
2 the formula:



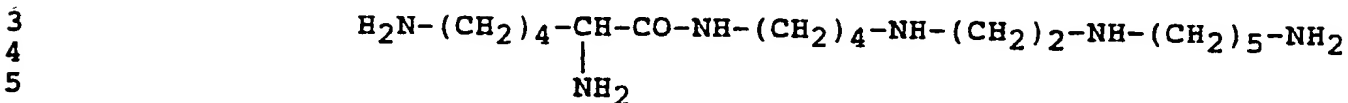
1 26. The method of claim 1 wherein said polyamine has
2 the formula:



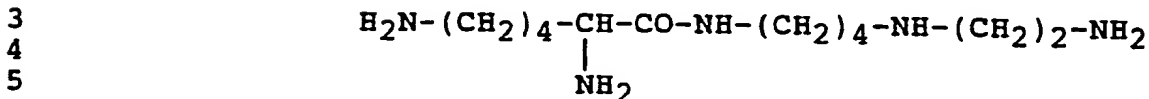
1 27. The method of claim 1 wherein said polyamine has
2 the formula:



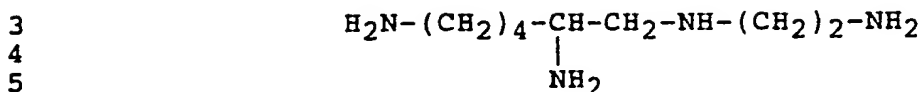
1 28. The method of claim 1 wherein said polyamine has
2 the formula:



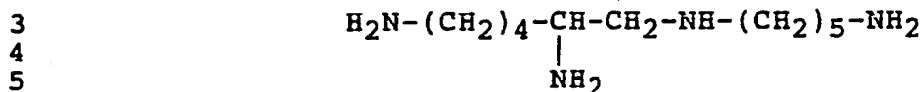
1 29. The method of claim 1 wherein said polyamine has
2 the formula:



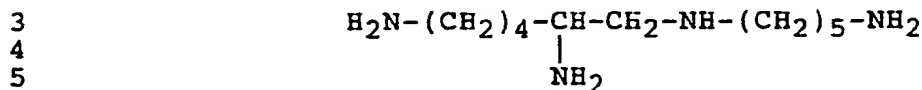
1 30. The method of claim 1 wherein said polyamine has
2 the formula:



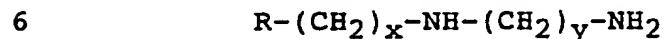
1 31. The method of claim 1 wherein said polyamine has
2 the formula:



1 32. The method of claim 1 wherein said polyamine has
2 the formula:



1 33. A method for blocking calcium channels resistant
2 to blockage by dihydroxypyridines, alcohols and conotoxin,
3 comprising exposing a cell membrane possessing said channels to
4 a nonaromatic polyamine calcium-channel blocking compound
5 within the formula (I):



7 wherein y is an integer from 1 to 15;

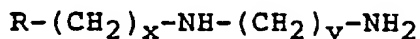
8 x is an integer from 0 to 15;

9 R is a nonaromatic organic group containing at least one
10 amino, imino, amido, or imido group and/or may be appended
11 to the rest of the formula via the group $-\text{CX}_2-\text{O}-\text{NH}-$ wherein
12 X is hydrogen or one of the X's is NH_2 with the proviso
13 that

14 the distribution of at least one of the methylene groups or
15 nitrogen atoms about the molecule of said polyamine is
16 asymmetric and that the compound contains at least three
17 nitrogen atoms;

18 said polyamine being present in an amount effective to block
19 calcium conductance attributable to said channels.

34. A method for preventing the occurrence or diminishing the magnitude of transmitter release resulting from the activation of neuronal calcium channels in a synapse, said channels being dihydropyridine-resistant, alcohol-resistant and conotoxin-resistant, the method comprising exposing said synapse to a nonaromatic polyamine calcium-channel blocking compound within the formula (I):



wherein y is an integer from 1 to 15;

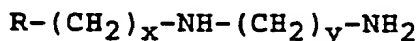
x is an integer from 0 to 15;

R is a nonaromatic organic group containing at least one amino, imino, amido, or imido group and/or may be appended to the rest of the formula via the group $-CX_2-O-NH-$ wherein X is hydrogen or one of the X's is NH_2 with the proviso that

the distribution of at least one of the methylene groups or nitrogen atoms about the molecule of said polyamine is asymmetric and that the compound contains at least three nitrogen atoms;

said polyamine being present in an amount effective to block calcium conductance attributable to said channels.

35. A method for activating calcium channels resistant to blockage by dihydropyridines, alcohols and conotoxin, comprising exposing a cell membrane possessing said channels to a nonaromatic polyamine calcium-channel activating compound within the formula (I):



wherein y is an integer from 1 to 15;

x is an integer from 0 to 15;

R is a nonaromatic organic group containing at least one amino, imino, amido, or imido group and/or may be appended to the rest of the formula via the group $-CX_2-O-NH-$ wherein X is hydrogen or one of the X's is NH_2 with the proviso that

the distribution of at least one of the methylene groups or nitrogen atoms about the molecule of said polyamine is

16 asymmetric and that the compound contains at least three
17 nitrogen atoms;
18 said polyamine being present in an amount effective to increase
19 calcium conductance attributable to said channels.

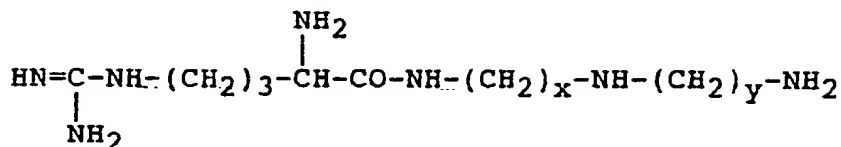
1 36. A method for enhancing transmitter release the
2 magnitude of transmitter release resulting from the activation of
3 neuronal calcium channels in a synapse, said channels being
4 dihydropyridine-resistant, alcohol-resistant and conotoxin-resis-
5 tant, the method comprising exposing said synapse to a nonaromatic
6 polyamine calcium-channel activating compound within the formula
7 (I):

8 $R-(CH_2)_x-NH-(CH_2)_y-NH_2$
9 wherein y is an integer from 1 to 15;
10 x is an integer from 0 to 15;
11 R is a nonaromatic organic group containing at least
12 one amino, imino, amido, or imido group and/or may be
13 appended to the rest of the formula via the group $-CX_2-O-$
14 $NH-$ wherein X is hydrogen or one of the X's is NH_2 with the
15 proviso that
16 the distribution of at least one of the methylene groups or
17 nitrogen atoms about the molecule of said polyamine is
18 asymmetric and that the compound contains at least three
19 nitrogen atoms;
20 said polyamine being present in an amount effective to increase
21 calcium conductance attributable to said channels.

22 37. A nonaromatic polyamine compound of the formula (I):
23 $R-(CH_2)_x-NH-(CH_2)_y-NH_2$
24 wherein y is an integer from 1 to 15;
25 x is an integer from 0 to 15;
26 R is a nonaromatic organic group containing at least one
27 amino, imino, amido, imido and/or may be appended to the
28 remainder of the formula via $-CX_2-O-NH-$ containing group
29 wherein X is hydrogen or one of the X's is NH_2 with the
30 provisos that

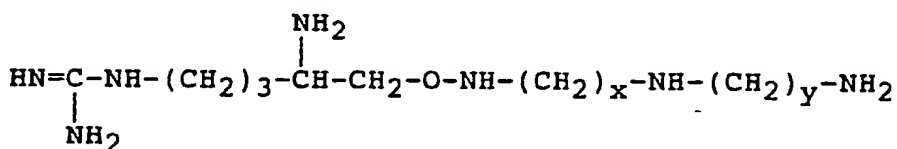
the distribution of at least one of the methylene groups or nitrogen atoms about the molecule of said polyamine is asymmetric and that the compound contains at least three nitrogen atoms; and that said compound is not $\text{H}_2\text{N}-(\text{CH}_2)_w-\text{NH}-(\text{CH}_2)_x-\text{NH}-(\text{CH}_2)_y-\text{NH}_2$ wherein w is an integer from 1 to 6.

38. The compound



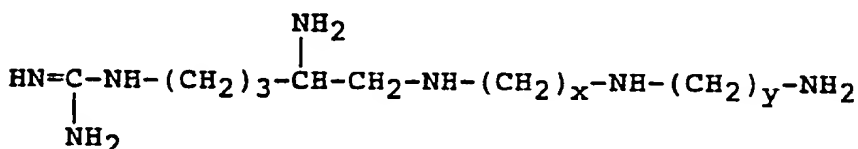
wherein each of x and y is independently 3 or 4.

39. The compound



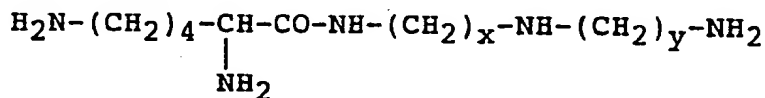
wherein each of x and y is independently 3 or 4.

40. The compound



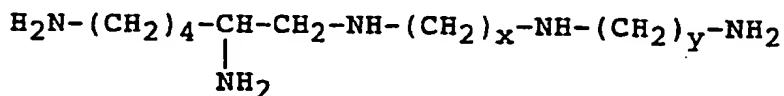
wherein each of x and y is independently 3 or 4.

41. The compound



wherein each of x and y is independently 3 or 4.

42. The compound



wherein each of x and y is independently 3 or 4.

43. The compound

68
69
70
71
72

$$\text{H}_2\text{N}-(\text{CH}_2)_4-\overset{\text{NH}_2}{\underset{|}{\text{CH}}}-\text{CH}_2-\text{O}-\text{NH}-(\text{CH}_2)_x-\text{NH}-(\text{CH}_2)_y-\text{NH}_2$$

wherein each of x and y is independently 3 or 4.

73 44. The compound
74 Arg-NH-(CH₂)₁₀-NH₂
75 wherein Arg is an arginine residue or a decarboxylated arginine
76 residue.

77 45. The compound
78 Arg-NH-(CH₂)₁₂-NH₂
79 wherein Arg is an arginine residue or a decarboxylated arginine
80 residue.

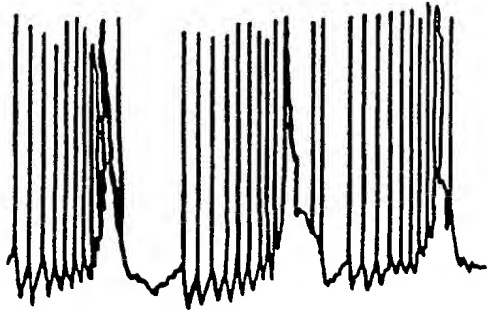


FIG. 1A

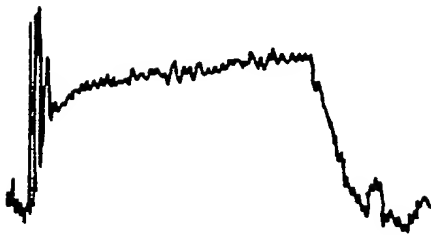


FIG. 1B



FIG. 1C

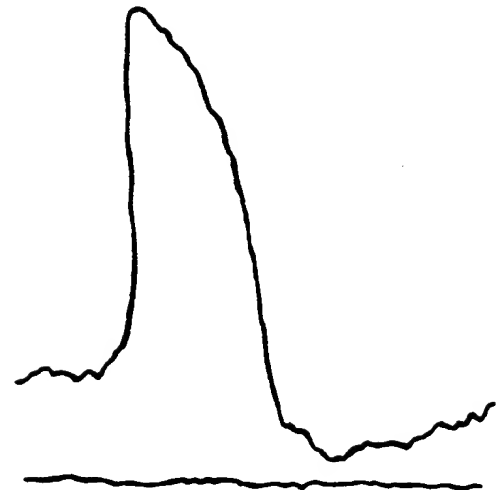
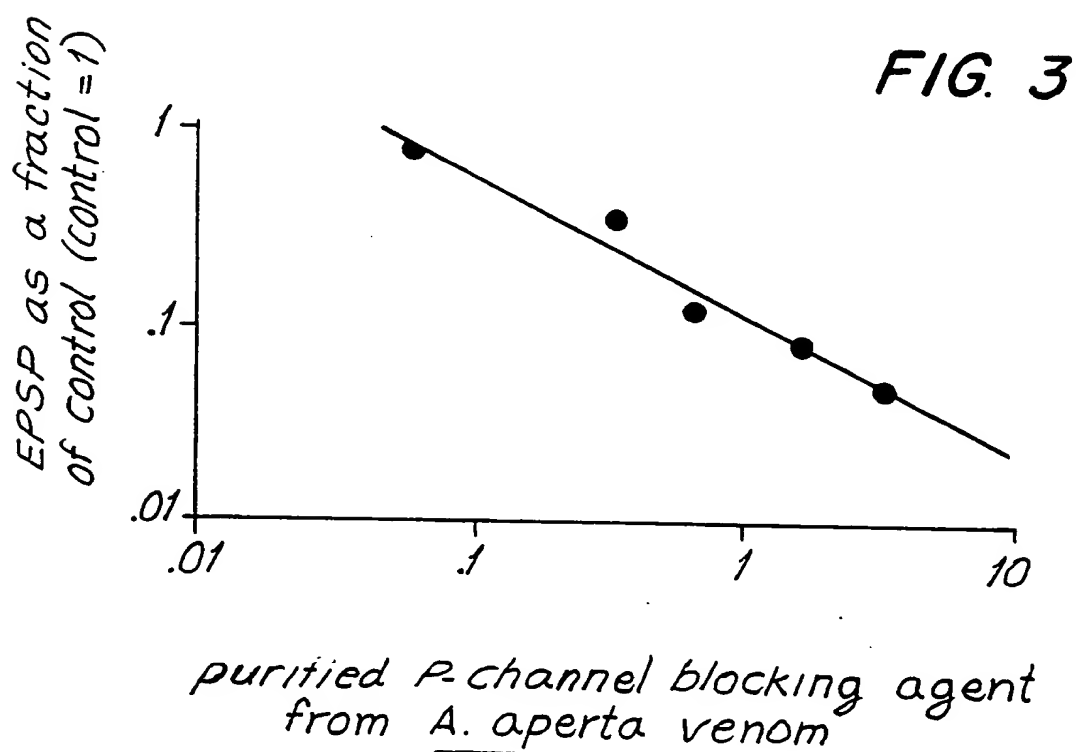
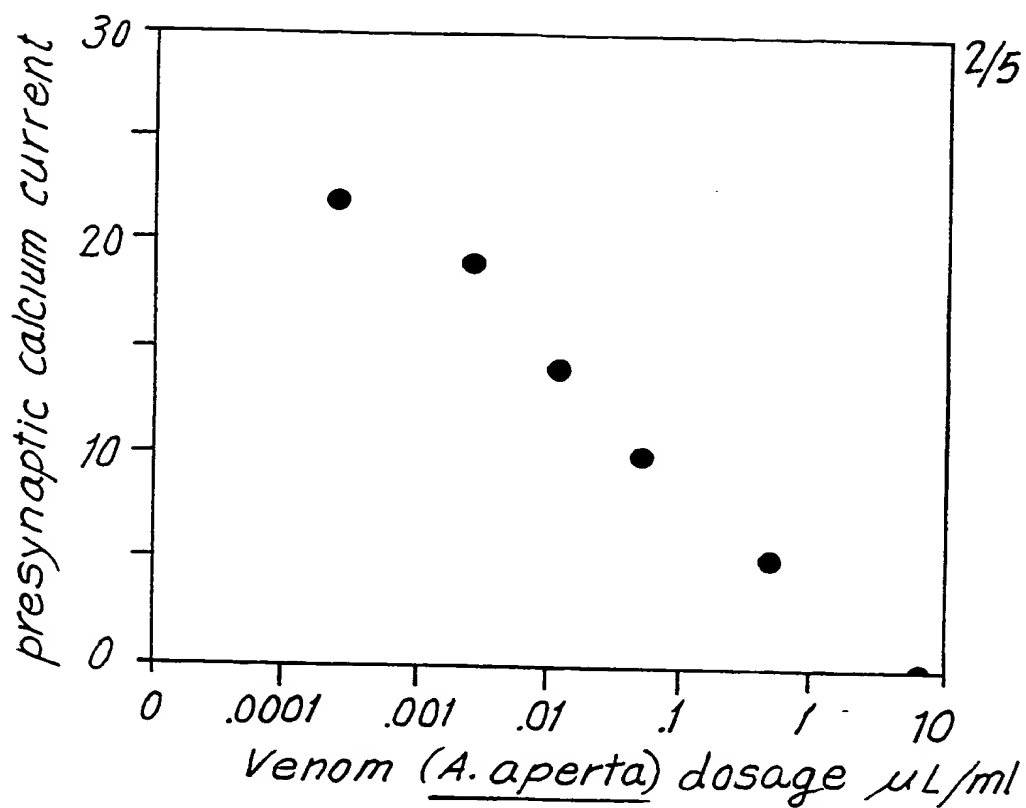


FIG. 1D

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FIG. 4

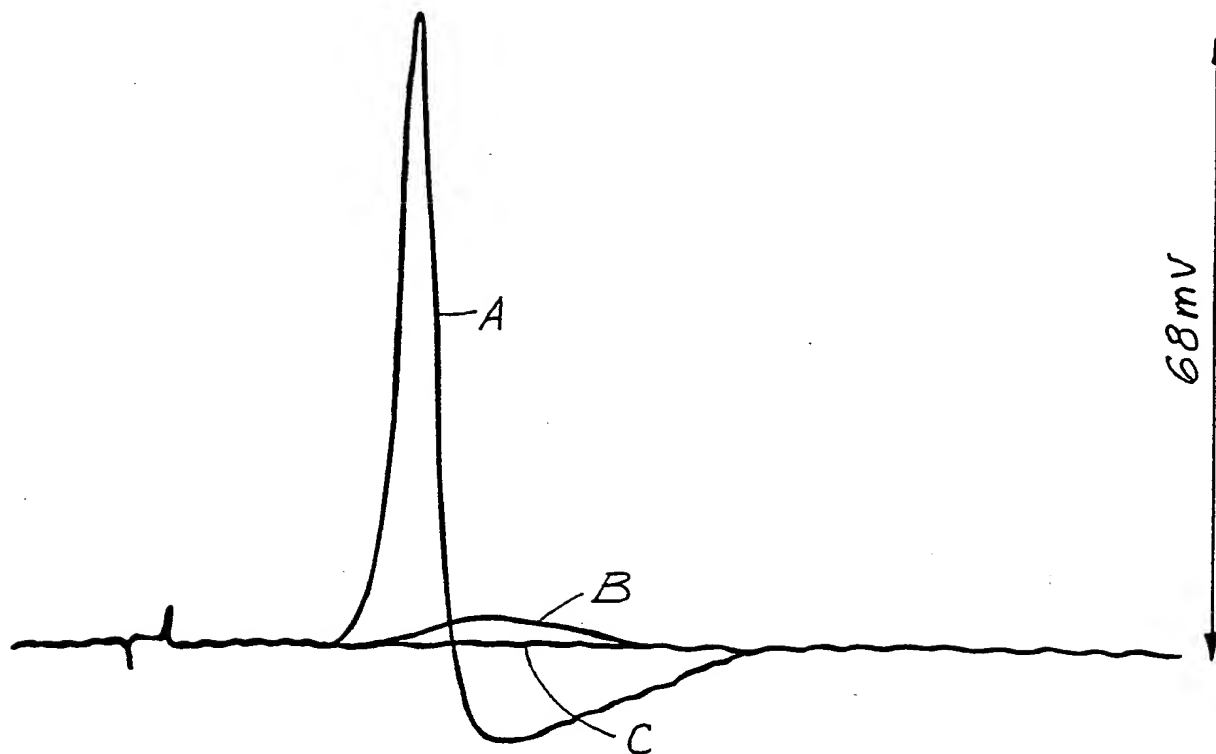
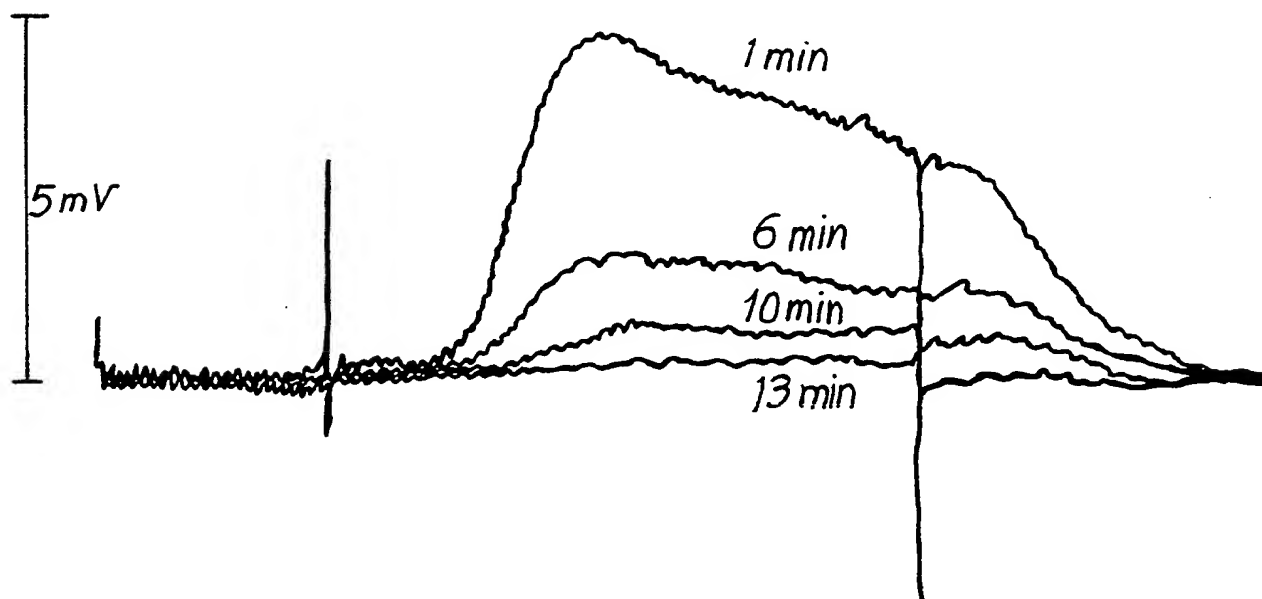


FIG. 6



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FIG. 5

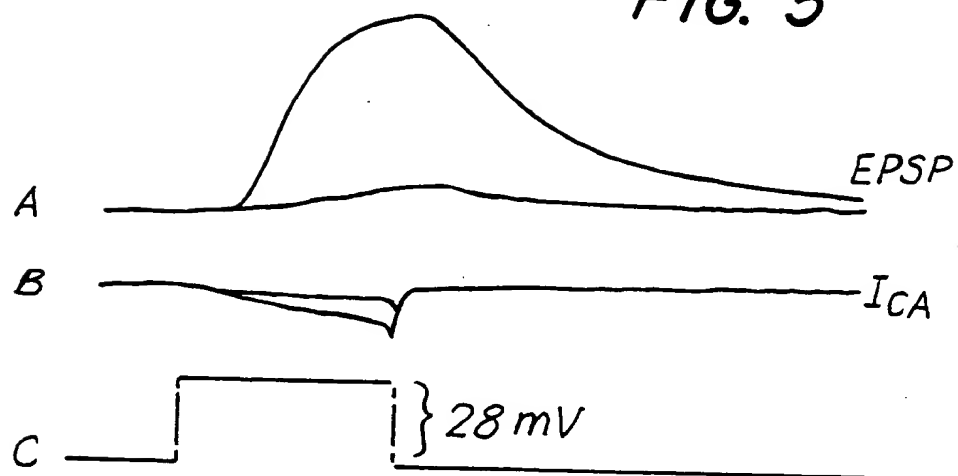


FIG. 7A



FIG. 7B



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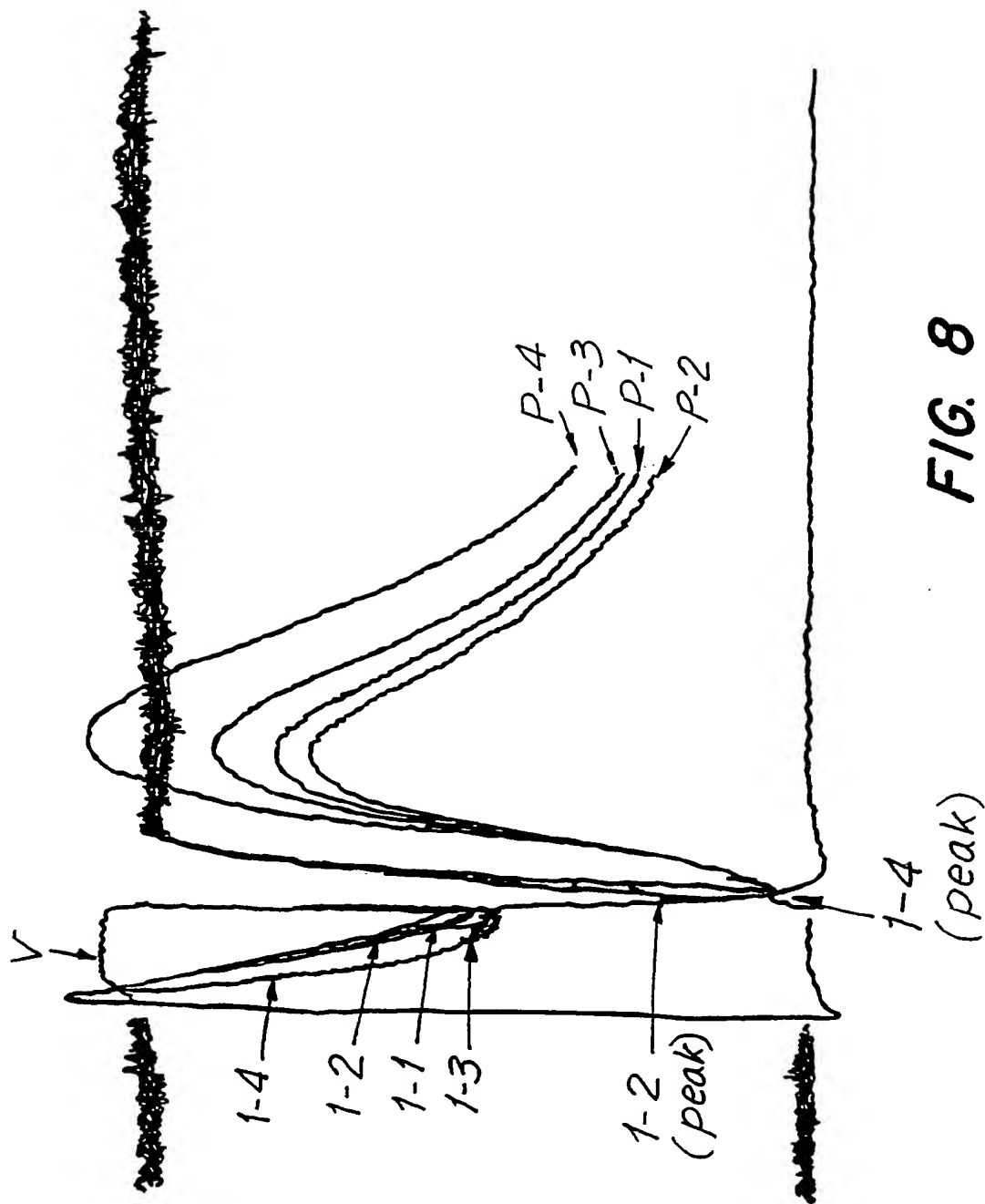


FIG. 8

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INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/03771

I. CLASSIFICATION OF SUBJECT MATTER (In several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07C 211/13, 211/33; C08H 1/00

U.S.C1.: 514/631, 636, 663, 673, 674; 564/243, 498

II. FIELDS SEARCHED

Minimum Documentation Searched *

Classification System

Classification Symbols

U.S. 424/537, 538; 530/350, 413; 514/631, 636, 663, 673, 674;
564/243, 498

Documentation Searched other than Minimum Documentation
to the extent that such documents are included in the fields searched

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X	The Journal of Biological Chemistry, Vol. 262, 1,33,37 No. 13, issued 05 May 1987, Palade, "Drug-induced Ca ²⁺ Release from Isolated Sarcoplasmic Reticulum", pages 6149-6154, see pages 6149-6154.	
A	Proc. Natl. Acad. Sci., Vol. 86, issued March 1989, Llinas, et al., "Blocking and isolation of a calcium channel from neuron in mammals and cephalopods utilizing a toxin fraction (FTX) from funnel-web spider poison", pages 1689-1693, see pp. 1689-1693.	
A	Annals New York Academy of Sciences, 560, issued 1989, Llinas's et al., "Voltage-dependent calcium conductances in Mammalian Neurons, The P Channel", pages 103-111, see pages 103-111.	

* Special categories of cited documents: ¹⁹

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search:

13 September 1990

International Searching Authority:

ISA/US

Date of Mailing of this International Search Report:

04 DEC 1990

Signature of Authorized Officer ²⁰

John Johnston
John Johnston